METHIONINE AND BROILER HEALTH

BY

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DISSERTATION

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ABSTRACT

Innovations directed toward improving broiler health while maintaining efficiency are critical to meeting industry demands. Although substantial advancements in poultry rearing have been made in the past 50 years, unavoidable events which activate the immune system result in reductions in production efficiency and economic loss to the producer. Recently, novel diet formulations targeted at improving broiler efficiency and immune function have been investigated. Nutritional strategies are practical, easily understood by producers, relatively cheap to implement without major structural changes, and generate impactful results. Amino acids have known effects on the immune system. Most of the requirements for amino acids are estimated from models using data obtained from un-challenged broilers where pathogen exposure is minimized. Though adequate for growth, these estimates may not reflect the bird's needs for both optimal growth and immune function. Methionine, the first limiting amino acid for growth in a standard broiler diet, is used in many cellular activities, including methylation, sulfur donation, pyruvate production, polyamine generation, formylmethionine formation, and is a precursor to cysteine and the potent anti-oxidants, Tau and glutathione. To determine if these immune-related functions of Met affect its requirement levels in the diet during challenged conditions and impact broiler immune function, a total of 3 experiments were conducted to investigate the relationship between dietary Met and broiler health. First, it was necessary to establish a diet formulation that permits manipulation of dietary Met and cysteine levels capable of estimating the requirement of 2 Met sources, their relative bioefficacy, and their impact on organ growth and development. To accomplish this aim, 2 Met dose-titration experiments were conducted using DL-Met and DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) as Met sources. For Experiment 1, 7 low-CP dietary treatments were fed during the grower phase (d 10 – 24): low total sulfur amino acid

(LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). For Experiment 2, 12 low-CP dietary treatments were fed to broilers during the grower phase (d 10 – 24): LTSAA, Met0, and Met0 supplemented with graded DL-Met (0.072% increments; Met1 – Met5) or DL-HMTBA (0.103% increments; HMTBA1 – HMTBA5). Growth performance was increased ($P < 0.001$) with Met supplementation. The LTSAA and Met0 differed for growth performance during d 10 – 24, but only during Experiment 2. From the fitted 1-slope broken-line regression analysis, the estimated requirement of supplemental DL-Met ranged from 0.18% – 0.19% (0.33% – 0.34% total Met) for BW gain and 0.12% – 0.15% (0.27% – 0.30% total Met) for gain:feed. From the same model, estimated requirements for supplemental DL-HMTBA were 0.22% (plus 0.15% Met) for BW gain and 0.17% (plus 0.15% Met) for gain:feed. The bioefficacy of DL-HMTBA relative to DL-Met was estimated using a nonlinear, exponential regression model and was 78% for BW gain and 82% for gain:feed. Absolute and relative gastrointestinal and immune organ weights and lengths were influenced by Met supplementation ($P <0.048$). Relative liver weight decreased ($P < 0.001$) in diets supplemented with at least Met2 or HMTBA3 and above compared with diets devoid of Met supplementation. A final experiment was conducted to examine the impact of dietary Met on the growth and immune performance of broilers and the requirement of supplemental DL-Met during an acute *in vivo* immune challenge. Broilers were fed 1 of 7 low-CP experimental grower (d 10 – 28) dietary treatments that included a low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5) and challenged *in vivo* intraperitoneally (i.p.) with lipopolysaccharide (LPS) at 2.0 mg/kg of BW on d 25. Growth performance was improved ($P < 0.001$) with the addition of supplemental DL-Met

before (d 10 – 24), during (d 24 – 26), and after (d 26 – 28) the *in vivo* LPS challenge. The estimated requirement of supplemental DL-Met from the 1-slope broken-line regression analysis numerically decreased for BW gain and gain:feed during the *in vivo* LPS challenge (d 24 – 26) by about 7% and 39%, respectively, compared with the requirement of supplemental DL-Met during the pre-challenge period (d 10 – 24). Dietary treatment had no impact ($P > 0.05$) on broiler body temperature. The ratio of heterophils to lymphocytes (H:L) decreased when at least Met3 or above was fed to broilers at all time-points following the *in vivo* LPS challenge. Additionally, dietary treatment had no impact ($P > 0.05$) on isolated splenic macrophage IL-6 production during an *ex vivo* stimulation by LPS assay. Absolute liver DM weights were impacted ($P < 0.001$) by dietary treatment at -24, 5, 24, and 72 hours post-stimulation (HPS), while relative liver DM weight was impacted ($P < 0.010$) only at -24, 5, and 72 HPS. Assorted main effects of dietary treatment, HPS, supplemental L-Cys, and supplemental DL-Met and their interaction effects on hepatic and splenic cytokine mRNA expression of *IL-6, IL-1β*, and *IL-10* were observed, with pro-inflammatory cytokines appearing more influenced by dietary Met than anti-inflammatory cytokines. Overall, this research demonstrates the importance of Met in maintaining broiler health during an immune challenge. Specifically, impacts made by the amino acid Met may occur during the onset of the innate response and enhance liver efficacy.

I want to dedicate this dissertation to my parents, Debbie and Mike Oelschlager, and Albert Towers, my sources of inspiration.

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Introduction

Diets are the main economic cost in U.S. broiler production. Protein and amino acid nutrition is an essential component of producing lean muscle on meat-type broiler chickens. Whereas environmental conditions have been optimized for large-scale broiler production, there remain unavoidable situations where disease challenges reduce the efficiency of production. Developing dietary strategies to reduce the incidence, severity, or duration of a disease challenge provides immediate benefits to production and can be easily understood and implemented by chicken producers. Most of the nutritional science that has been produced in the past century has been with healthy birds, and the requirements for amino acids are based mainly on growth performance parameters. However, we know that nutritional requirements change quickly and dramatically upon activation of the immune system. How the provision of specific dietary amino acid profiles can benefit the immune response and complement pharmaceutical strategies to maintain a healthy and productive flock remains to be elucidated.

Inadequate diets can impact a bird's ability to facilitate a robust immune response. The amino acid methionine is critical in broiler nutrition as the first limiting amino acid in a corn-soybean meal diet and is diverse in its biological use. Some of methionine's involvement in immune function includes cellular processes like polyamine synthesis, DNA methylation, biosynthesis of cysteine, taurine, and glutathione, immune proteins, and cells. Although scientists have investigated ways methionine may aid broiler health, there remain areas of inconsistency, and the influence methionine may have on a broiler's immune response remains poorly understood. Further research into methionine's role during an immune response can help

characterize the nutritional requirements of a broiler during immune stress and may prevent overt inflammation, limit unnecessary energy expenditures, and promote a consistent rate of growth.

The relationship between avian health and amino acids

Overview of the avian immune system

To achieve optimal health, a broiler chicken requires a functional immune system that allows it to protect and defend itself from immune challenges, such as heat stress or disease, which have detrimental effects on the growth performance of broilers (Zhang et al., 2015; Rochell et al., 2016a). A robust immune response from a bird's immune system is an orchestration of processes coordinated by the innate and adaptive arms of immunity. The innate immune system represents the "first line of defense" for a host and relies on a system of barriers, both physical (e.g., respiratory and intestinal lining, skin, feathers) and chemical (e.g., acute phase proteins, complement cascade) to prevent immune stressors from overwhelming the bird (Juul-Madsen et al., 2012). An immune barrier of particular importance to broilers is gut-associated lymphoid tissue (GALT) because of their vulnerability to gut-pathogen exposure (i.e., consumption of contaminated litter, feed, water), and it is made up of the Meckel's diverticulum, cecal tonsils, Peyer's patches, bursa of Fabricius, and clusters of lymphocytes (Lillehoj and Trout, 1996; Yegani and Korver, 2008). The GALT also includes the mucosal lining of the intestine, a multifunctional barrier that aids in warding off a pathological assault by capturing microorganisms in its outermost mucosal layer and preventing penetration to the vulnerable epithelium beneath (Broom, 2018). If barriers are compromised, an event usually triggered by pattern recognition receptors, the innate immune system initiates a response and expeditiously deploys innate immune cells like granulocytes, monocytes, macrophages, and natural killer (NK) cells to participate in mounting the response (Juul-Madsen et al., 2012).

Innate immune cells individually take part in various roles within the immune response. However, their production of cytokines is integral to a robust and efficient response that helps the bird overcome infection. Cytokines are small proteins that act as messengers and regulators during an active immune response by signaling to surrounding cells and recruiting necessary resources to the site of inflammation (Klasing, 1994; Kogut, 2000). Changes in cytokine production because of immune stimulation can potentially cause unwanted impacts to behavior. Sickness behavior, albeit necessary for overcoming infection, causes fatigue and reduced feed intake and is induced by pro-inflammatory cytokine activity in the brain (Kelley et al., 2003). The acute phase response, characterized by the rapid change in the production of acute-phase proteins by hepatocytes in the liver, is also regulated by cytokines, specifically the pro-inflammatory cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) (Chamanza et al., 1999). In chickens, during an active acute phase response, the acute phase proteins α1-acid glycoprotein (AGP), ceruloplasmin, serum amyloid A, and transferrin increase by 1- to 10- fold, and each maintains a distinct purpose in facilitating the response (Cray et al., 2009). A broiler's innate immune response acts swiftly to protect itself from immune stimuli but, consequently, is non-specific. In contrast, the adaptive immune response is delayed but can target specific immune stimuli and respond accordingly.

These two arms of immunity, innate and adaptive, are highly integrated. The sequence of events in an innate response alone may not put the host in the clear. As a result, the development of the adaptive antigen-specific memory immune response is initiated. This arm generates immunological memory, allowing for a more efficient response when encountering an immune stimulus for a second time. The adaptive immune system is made up of both humoral and cellular responses. The humoral response is orchestrated by B-lymphocytes (B-cells) produced

by the bursa of Fabricius, while the cellular response is mediated by T-lymphocytes (T- cells) and regulated by the T-cell maturation hub, the thymus (Sharma, 1991). The type of immune stimulus dictates the required profile of adaptive immune cells during the response, and the activity of T-cells influences the reaction of B-cells (Erf, 2004; Jeurissen et al., 2000). The adaptive response works in concert with the innate response to facilitate a complex, rapid, and regulated response for overcoming immune challenges.

An immune response is essential to recovering from infection and immune stress, but activating, utilizing, and maintaining the response affects metabolic homeostasis within the bird. Immune challenges are a unique and complex period in a bird's life because even marginal disruptions in growth can impact profitability due to the short production time needed by broilers to achieve market weight (< 6 weeks). A broiler's appetite is decreased immediately following an activated immune system, while the demand to generate vital innate immune cells increases (Chamanza et al., 1999; Rochell et al., 2016a). This imbalance (i.e., inflammation) creates a catabolic environment, and protein degradation following immune activation significantly increases within the spleen, thymus, and muscle of broiler chicks (Klasing and Austic, 1984a). The acute phase response is a large sink for free amino acids resulting from immune-related protein degradation (Barnes et al., 2002). During immune activation, nutrients are prioritized for maintenance and the immune system requirements, and proceed towards growth only after those requirements are met (Humphrey and Klasing, 2004). While some organs and tissues undergo protein degradation in response to immune activation, others respond by increasing protein synthesis. Chicks increase protein synthesis in immune-related organs like the liver, bursa, spleen, and thymus while diminishing protein synthesis in muscle during non-infectious immune challenges (Klasing and Austic, 1984b).

Indeed, protein degradation produces amino acids for energy use and generates substrates that can be used for immune cells and proteins (Beisel, 1988). However, from a producer standpoint, preference is placed on free amino acids to be sourced via the diet, not by protein degradation, as the need for the production of poultry meat drives the broiler industry. During a mounted immune response, growth is impaired, of which 70% is attributed to a reduction in feed consumption (Klasing and Leshchinsky, 1999). Vaccination programs as an alternative to antibiotic use are becoming more common within the broiler industry, but their use relies on an effective immune response to generate immunological memory, and with it can come decreased growth performance. Immunity-induced decreases in growth resulting from some vaccines may not be recovered by the time the bird is processed (Williams, 1998). Vaccination against coccidiosis upon hatch coupled with access to starter diets containing high amino acid density improves broiler growth performance and carcass traits (Cloft et al., 2019). Access to adequate dietary amino acids was needed for the coccidiosis vaccinated broilers to perform optimally.

A method for reducing the occurrence, severity, or extent of an immunological challenge is dietary intervention. Nutritional strategies are practical to use, easily understood, and relatively cheap for producers while having the potential to generate impactful results. The current nutrient recommendations being utilized in the broiler industry are estimated using models based on optimum growth and feed efficiency and most often calculated in environments where exposure to pathogens is minimized. Though adequate for growth, these calculations may not align with the requirements needed for overall bird health. Considering bird health, or both growth and immune performance better reflects an industry setting where birds routinely encounter pathogens and environmental stressors.

A nutrient recommendation of particular importance in poultry diets is protein because it is the costliest component of a standard commercial broiler diet. The dietary protein level is also crucial because of the pressure brought on by a demand for a more sustainable animal agriculture industry. Within the poultry industry, research investigating the impact of low crude protein diets supplemented with crystalline amino acids for the betterment of the environment has been expanding (Hilliar et al., 2020; van Emous et al., 2019). It is estimated that premature deaths due to total atmospheric fine particulate matter ($PM_{2.5}$) from food production can be reduced by roughly 40% when switching from red meat consumption to poultry consumption and by about 20% with enhanced manure management (Domingo et al., 2021). As the body of research on supplementation of crystalline amino acids to broiler diets has increased, so has their commercialization and economic benefit of use by producers, with four to five amino acids now feasibly available for supplementation (Elwinger et al., 2016). The current literature and dietary guidelines for amino acid recommendations utilized by the broiler industry may be insufficient to support optimal bird health. Developing and maintaining a functional immune system requires resources needed by the bird that are different than what is needed to promote growth. Supplementing diets with amino acids during immune challenges is a nutritional approach that has proven beneficial in promoting broiler health (Emadi et al., 2011; Jahanian, 2009; Ruiz-Feria and Abdukalykova, 2009; Sigolo et al., 2017).

Supporting the avian immune system with amino acids

Dietary protein is made up of individual amino acids. Some level of intact protein is required in a poultry diet for optimal growth, making it an essential nutrient, but the requirement for protein is derived from the biological need for individual amino acids (NRC, 1989; NRC, 1994). Of the roughly twenty amino acids required for producing various proteins in an animal,

ten are considered essential amino acids in chicks, or those which must be obtained via the diet as the animal is incapable of producing them *de novo* (Karau and Grayson, 2014). Indeed, the ten essential amino acids are integral to broiler health, but those that are both essential and limiting amino acids are given special consideration when formulating broiler diets. A limiting amino acid can be explained by implementing Liebig's Law of the Minimum. Originally applied to crop growth, Liebig's law states that "essential material available in amounts most closely approaching the critical minimum needed will tend to be the limiting one" (Danger et al., 2008). When applying this law to amino acid nutrition in poultry, the amino acid at the lowest concentration in a diet to support a metabolic processes will dictate the rate of the metabolic process, irrespective of the level of other amino acids in the diet.

In most standard corn-soybean meal-based broiler diets, methionine is recognized as the first limiting amino acid for growth (Fernandez et al., 1994). Similarly, methionine is also most limiting in low crude protein corn-soybean meal diets (16% crude protein) (Edmonds et al., 1985). When a diet is devoid of protein, a bird must rely on the catabolism of body protein to meet amino acid requirements, effectively diminishing the capacity for growth because endogenous amino acids are being diverged towards maintenance. Dietary addition of methionine, cysteine, or a combination thereof equally lessens weight change and improves protein accretion when broilers are fed diets devoid of intact protein, revealing that the limiting nature of methionine is brought on by the demand for cysteine in regards to usage for endogenous amino acids (Webel and Baker, 1999). Though cysteine is the driving factor behind methionine's classification as the first limiting amino acid, cysteine is not generally recognized as a limiting amino acid of concern when formulating a standard corn-soybean meal-based broiler diet, assuming supplemental dietary methionine is provided at a level that satisfies the

requirement for both methionine and cysteine. Because of methionine's ability to furnish cysteine, the requirement of methionine is often reported as a requirement for methionine + cysteine or total sulfur amino acids (TSAA).

If the demand for the most limiting amino acid in a diet is not met, the capacity for protein synthesis is diminished; therefore, it is crucial to ensure the levels of these amino acids are being met, not just for optimal growth but optimal immune performance. Current literature is focused on the use of novel feed additives as a means of dietary intervention, but re-evaluating the dietary requirements for amino acids, requirements that have been utilized in the U.S. since 1954 when the first Poultry NRC was established, is beneficial for supporting and promoting bird health during times of immune stress (Elwinger et al., 2016). Because of its vulnerability to gut-pathogen exposure, the GALT is an area of focus for assessing feed additive effectiveness. Expression of MUC2 mRNA, the gene encoding the principal protein of gel-forming mucin within the mucus layer, is used as a biomarker to quantify the integrity of the gut barrier in chickens (Chen et al., 2015; Jiang et al., 2013). Laying hens housed in high temperatures (30 ± 5°C) and relative humidity (85 ± 3%) for nine weeks demonstrated a linear increase in MUC2 mRNA expression within the jejunum and ileum as dietary L-threonine concentrations increased (Azzam et al., 2011). Increasing concentrations of dietary L-threonine also improved antibody production following infection with Newcastle disease virus in chicks and restored diversity of intestinal bacteria in laying hens fed low crude protein diets (Bhargava et al., 1971a; Dong et al., 2017). Taken together, dietary L-threonine supports intestinal barrier integrity and microorganismal populations in the gut while also enhancing antibody production.

Another essential amino acid important to the immune system of chickens is arginine. Arginine is the principal precursor to nitric oxide, a defense molecule with anti-microbial activity

that serves as a vital component of innate immunity but is also toxic in excess and can cause unwanted damage to cells (Bogdan, 2015). Increasing dietary arginine improves the immune response to both viral and bacterial disease challenges in chickens (Lee et al., 2002; Takahashi et al., 1999; Tan et al., 2015; Zhang et al., 2017). Dietary supplemental arginine 2.5-times greater than the recommendations outlined in the Poultry NRC (1994) significantly increased serum levels of IgG and IFN-γ at 7, 14, and 21 days following a challenge with infectious bursal disease virus (Emadi et al., 2011). In the same study, tryptophan supplemented 2-times greater than recommendations in the Poultry NRC (1994) elicited similar effects on serum levels of IgG and IFN-γ following the infectious bursal disease virus challenge (Emadi et al., 2011). Tryptophan is an essential amino acid not often supplemented to poultry diets but is being reconsidered because of its role in metabolic regulation and metabolite synthesis (Fouad et al., 2021). Dietary supplemental tryptophan fed to broiler chicks at roughly 1.5-to-2-times the level recommended by the Poultry NRC (1994) enhanced the immune response to an infectious bursal disease virus challenge by increasing serum IFN-γ 21 days and serum IgG levels 7, 14, and 21 days post-challenge (Emadi et al., 2010).

Effector immune cells, like T- and B-lymphocytes, use glutamine for fuel during an inflammatory challenge, and as a result, glutamine is considered conditionally essential during this time (Gaber et al., 2017; Newsholme, 2001). Dietary glutamine supplemented at 10 g/kg reduced necrotic enteritis lesion scores in the jejunum and ileum (Xue et al., 2018). Glutamine supplemented at 10 g/kg of the diet significantly increased duodenal and jejunal villi height and concentrations of serum IgA and serum IgG at 7, 14, and 21 days of age and enhanced intestinal IgA at 21 days of age, suggesting glutamine may support the development of the immune system and integrity of the gut (Bartell and Batal, 2007). Glutamine presents an interesting opportunity

to support GALT health. Without immune stress, cells of the gut already utilize most dietary glutamine (Newsholme, 2001). In humans, glutamine supplementation is utilized to alleviate some intestinal diseases by protecting tight junctions and preserving mucosal integrity of the gut (Rao and Samak, 2012). Since enterocytes metabolize most dietary glutamine, a limited supply of glutamine may be available for use by immune cells in the GALT. The broiler gut is constantly exposed to immune stressors (i.e., dirty litter, feed, water, general curiosity to peck). Thus the use of dietary glutamine, an amino acid not routinely supplemented to a broiler diet, may offer additional support to immune cells of the GALT.

The requirements of some amino acids may adapt to the direct demand for nutrients to support the development and functionality of a broiler's immune system. Understanding these requirements in challenged conditions allows nutritionists to specifically feed a bird to its needs based on what we know about a specific amino acid and its response to a stressor. Because other essential amino acids have proved impactful to broiler health, methionine, the first limiting amino acid in standard poultry diets, warrants further investigation and discussion on how it may play an integral part in assisting the bird in developing a robust and adaptable immune system.

Dietary methionine

Metabolism and biological functions of methionine and its metabolites

As our knowledge on the importance of poultry nutrition and metabolism beyond growth has increased, we have uncovered the influence nutrition plays on immune-related biological functions. The metabolism of methionine and its metabolites are utilized in myriad reactions within a bird, increasing the likelihood of an event where the dietary level of methionine may influence immune development and function. Some of these metabolites include S-adenosylmethionine (SAM), choline, and betaine, in addition to other amino acids (collectively

known as the sulfur amino acids) like homocysteine, taurine, and cysteine, a precursor to the anti-oxidant molecule, glutathione (Wu, 2009).

Methionine metabolism begins with the conversion of methionine to SAM by the enzyme S-adenosylmethionine synthase (Parkhitko et al., 2019). The molecule SAM is most notable for its role as a major methyl donor for a diverse number of substrates like proteins, phospholipids, RNA, and DNA and the subsequent impact methylation has on cellular mechanisms (Lu, 2000; Zhang and Zeng, 2016). The methylation of DNA is crucial for regulating gene transcriptional activity of immune functions like proliferation, differentiation, and regulation of T and B cells, mediation of NK cells, production of pro-inflammatory cytokines, and complement activation (Suarez-Alvarez et al., 2012). In addition, SAM can also be decarboxylated and used to produce polyamines (Lu, 2000). Polyamines (e.g., putrescine, spermidine, and spermine) serve a role in the adaptive immune system by influencing T- and B-lymphocyte proliferation, in the innate immune system by signaling to some toll-like receptors for activation, and in the cell as a whole by facilitating growth (Handa et al., 2018; Hesterberg et al., 2018; Igarashi and Kashiwagi, 2000).

Another product of methionine metabolism, S-adenosylhomocysteine (SAH), can be further catabolized in a reversible reaction to form homocysteine and adenosine by the enzyme SAH hydrolase (Brosnan and Brosnan, 2006). In healthy cells, this reaction produces adenosine, a signaling molecule that can influence the response of the innate immune system by reacting with receptors on macrophages to promote the production of the anti-inflammatory cytokine interleukin 10 (IL-10) while reducing the production of pro-inflammatory cytokines interleukin 12 (IL-12) and TNF-α (Kumar and Sharma, 2009). Homocysteine has two main fates, depending on the status of the cell. When more methionine is needed (i.e., deficiency), homocysteine can be

converted back to methionine using one of two pathways, either with folate, vitamin B_{12}, and the enzyme methionine synthase or by the enzyme betaine-homocysteine methyltransferase with the addition of betaine (Lu, 2000; Ganguly and Alam, 2015). When methionine is adequate (i.e., sufficiency), homocysteine is transformed by the enzyme cystathionine β-synthase and serine in an irreversible reaction to cystathionine, and cystathionine is further catabolized to cysteine via the enzyme cystathionine γ-lyase (Brosnan and Brosnan, 2006; Ganguly and Alam, 2015).

Cysteine is a precursor to many essential molecules and is unstable (Wu, 2009). As a result, cysteine is quickly oxidized to form cystine (stable form), utilized in protein synthesis, converted to pyruvate, transformed to glutathione, or used to produced taurine (Brosnan and Brosnan, 2006; Wu, 2009). Taurine is a sulfonic acid that is not directly used in protein synthesis (i.e., not essential); however, taurine is a powerful anti-oxidant and may regulate and modulate the immune system (Surai et al., 2020). Japanese quail supplemented with taurine (0.05% taurine) have increased thymus and bursa organ weights when adjusted for BW, and increased foot web indexes to the immune stimuli phytohemagglutinin, an assay used to quantify the cellular response at the site of infection (Wang et al., 2009).

Glutathione is generated from the rate-limiting amino acid cysteine, γ-glutamylcysteine synthetase and glutathione synthetase enzymes, glutamate, and glycine, and serves many purposes in cells, but is most known for its utilization in oxidation-reduction reactions (Lu, 2000; Wu et al., 2004). Reduced forms of glutathione act as electron acceptors and can be oxidized into glutathione disulfide nonenzymatically when in the presence of oxidants like free radical reactive oxygen and nitrogen species (Wu et al., 2004). Under normal conditions, oxidants serve vital metabolic functions, but when left to accumulate, they can induce oxidative stress (Sies, 1997). Factors contributing to oxidative stress in broiler production include improper handling prior to

slaughter, consumption of oxidized oil, nickel chloride, or copper from the diet, and exposure to heat (Estévez, 2015). Oxidative stress increases susceptibility to disease and decreases performance, inevitably causing economic losses in poultry production (Estévez, 2015).

There is an entangled relationship between oxidative stress and immune reactivity. A meta-analysis of 16 papers from both wild and domesticated bird species found immune challenges to impact critical oxidative stress markers directly, highlighting the connection between immune stress and oxidative stress (Costantini and Møller, 2009). Nuclear factor-κB is likely implicated in this interconnection by being influenced by and a regulator for oxidative stress (Lingappan, 2018). This is of little surprise as Nuclear factor-κB is the primary group of transcription factors that mediate integral immune functions like production of cytokines, chemokines, and adhesion molecules, differentiation of T-cells, and signaling neutrophils to the site of inflammation (Liu et al., 2017). Because of this complicated relationship, dietary supplements that demonstrate both anti-inflammatory and anti-oxidant properties are highly sought after. Broilers stressed immunologically by coccidiosis or oxidatively by dietary inclusion of oxidized oil decreases levels of thiobarbituric acid reactive substances, a measurement of lipid peroxidation, in plasma with the inclusion of dietary methylsulfonylmethane (MSM) (Catalán et al., 2018; Rasheed et al., 2020a; b). The sulfur-containing compound MSM is used in human and companion animal nutrition as a dietary supplement for its anti-inflammatory, anti-cancer, and anti-oxidant characteristics (Butawan et al., 2017).

The biological roles of methionine and its subsequent metabolites in the health of a bird and their influence on the response to immune stimuli make methionine an ideal target for immune study. Typically, growth parameters are the primary data collected and analyzed to investigate the direct impact of novel feed technologies or practices on the broiler industry, as

these make the most meaningful gains to the producer. Nevertheless, without understanding the cellular impact on the broiler, a novel feed technology may not be applied to its best ability. Insight at the cellular level allows for better use of technology by focusing on a specific known mode of action and exploiting its positive impact, and advancing our knowledge of broiler nutritional requirements that better reflect an industry setting where known immune stimuli exist.

Dietary sources of methionine

Because the metabolism of methionine produces cysteine *de novo*, practical broiler diets often express the level of methionine in a diet as "methionine + cysteine" or TSAA. Indeed, dietary methionine can be used to fulfill the requirement for methionine and cysteine, but the general interpretation that the single reported value will be split equally for methionine and cysteine is a simple explanation to a rather complex relationship between the two amino acids. Characterizing this relationship is crucial for understanding the optimal level of methionine required and has been extensively discussed (Baker and Dilger, 2009). In brief, the production of cysteine from methionine via the transsulfuration pathway (i.e., from homocysteine) is 100% efficient, but in this conversion, one mmol of methionine weighing 149.2 mg produces just 121.2 mg of cysteine (Baker and Dilger, 2009). The metabolic conversion of methionine only yields 81% as much cysteine on a molar basis; consequently, feeding methionine alone to satisfy the requirement for methionine and cysteine may underfeed methionine. Feeding methionine alone to fulfill the requirement for methionine + cysteine will yield a higher minimum requirement for methionine compared to meeting the requirement with the addition of both methionine and cysteine to the diet (Baker and Dilger, 2009). Methionine titration in purified diets containing insufficient or adequate cysteine elicits estimated methionine requirements of 700 and 314 mg $\cdot$ $kg^{-1} \cdot d^{-1}$ (milligrams per kilograms per day), respectively when based on body weight gain

(Baker and Dilger, 2009). From these results, a simple approach estimates the requirement of cysteine to be 386 mg · kg^{-1} · d^{-1} if 314 is subtracted from 700; however, this is an erroneous estimation, evidenced by a third assay in which cysteine is fed at different dosage levels, and methionine is set at 314 mg · kg^{-1} · d^{-1} and the requirement was found to be 314 mg · kg^{-1} · d^{-1}, 81% of the initial false 386 mg · kg^{-1} · d^{-1} estimation (Baker and Dilger, 2009). Suppose a bird is fed methionine solely to satisfy the requirement for both methionine and cysteine. In that case, more will be needed (i.e., 700 mg · kg^{-1} · d^{-1}) than if methionine is supplemented with cysteine in excess (314 + 314 = 628 mg · kg^{-1} · d^{-1}) (Baker and Dilger, 2009). The addition of supplemental cysteine to the diet effectively spares the use of methionine, but its addition can also change the extent of the response in a methionine requirement study for growth parameters, demonstrating yet another layer of complexity to investigating methionine requirements (Baker and Dilger, 2009). With this information in mind, one can speculate that the same altered response might be observed when assessing immune parameters instead of growth, so it is essential to consider the level of cysteine when determining the requirement for methionine. To avoid discrepancies in methionine requirement estimations, the level of cysteine should be fed in excess or at least sufficiently, and at a fixed level across diets to ascertain the requirement of methionine needed for the bird truly.

When comparing methionine or TSAA (i.e., methionine + cysteine) requirement studies, it is crucial to consider the level of digestible cysteine in a diet, especially considering within the broiler industry, there are multiple methionine sources available and are nearly always supplemented into a commercial broiler diet. The most common dietary sources of methionine are DL-methionine and DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA). For years, there have been disagreements within the literature as to how both of these sources meet the

requirement for methionine, as the method and enzymes needed for metabolism to L-methionine (the form considered 100% useable in the cell) differ, and the amount found in each source, DL-methionine vs. DL-HMTBA, can also vary among products (Dibner, 2003; Vázquez-Añón et al., 2006). The precursor to L-methionine in both metabolic pathways of DL-methionine and DL-HMTBA is 2-keto-4-(methylthio) butyric acid (keto analog of methionine), and research on its use as a source has also been conducted, but not to the extent of the other two (Dilger et al., 2007a). A multiple linear regression on the use of DL-methionine and DL-HMTBA (62 references) in broiler nutrition trials determined that when the two sources are fed on an equimolar basis, there is no difference in performance observed (Vázquez-Añón et al., 2006). Contrarily, a meta-analysis (27 references) of the bioefficacy of DL-HMTBA relative to DL-methionine indicated that DL-HMTBA is 81% and 79% effective as DL-methionine for average daily gain and feed efficiency, respectively (Sauer et al., 2008).

Cysteine is an amino acid not routinely included in commercial broiler diets but is utilized in research settings for determining methionine and TSAA requirements. Researched sources of supplemental dietary cysteine in broilers include L-cysteine, L-cystine, and N-acetyl-L-cysteine, a non-methionine-derived precursor to L-cysteine (Dilger and Baker, 2007). Dietary L-cysteine fed in excess at 7.5 times the requirement causes mortality in as little as five days after feeding (Dilger et al., 2007b). Based on these results, it is possible to confound research results by feeding dietary cysteine levels that are too high, due to its toxicity.

Methionine and the health of broilers

Methionine and immune development and function in broilers

The first half of a broiler's life is a crucial developmental stage for the bird. An adverse condition, like deficiency of an amino acid, can impair the growth and development of a bird,

including its immune organs (Konashi et al., 2000). Immune organs and tissues of the avian lymphatic systems can be classified as either primary (e.g., bursa of Fabricius and thymus), where lymphocytes are made, or secondary (e.g., spleen, lymph nodes, and mucosal tissues like the gut- eye- nasal- and skin-associated lymphoid tissue), where lymphocytes are exposed to antigens (Olah et al., 2012). The weights of primary and secondary immune organs adjusted for bird body weight are often quantified as a measure of immune development or efficacy in broilers (Bartlett and Smith, 2003; Kautzman et al., 2017; Shinde Tamboli et al., 2017).

Supplemental methionine fed to birds has a significant impact on immune organ weights. Birds receiving supplemental DL-methionine in the first seven days post-hatch display increased weights (mg/100g of BW) of the immune-related organs bursa, thymus, and spleen at 28 days post-hatch, while birds receiving supplemental DL-methionine at 29-36 days post-hatch showed no impact to the weight of immune-related organs at 50 days post- hatch (Al-Mayah, 2006). Male layer breeders fed supplemental DL-methionine at 171 or 243% above Poultry NRC nutrient recommendations (1994) for the first four weeks post-hatch and stimulated with sheep red blood cells displayed increased weight (g/kg of BW) of the thymus and bursa (Deng et al., 2007). A deficiency in methionine also impacts immune organ metrics. Supplemental DL-methionine fed at 50% during 0-3 weeks of age and at 75% during 3-6 weeks of age of the Poultry NRC (1994) requirements for broilers significantly decreased the weight (g/kg of BW) of the thymus at 21, 28, 35, and 42 days of age compared to a control, methionine adequate diet (Wu et al., 2012).

The liver, a secondary lymphoid organ because of its ability to process antigens, is also a significant immune organ for its role in the rapid production of the acute phase, anti-microbial, and complement proteins needed in immune reactions (Robinson et al., 2016). There is little

research on the fluctuation of liver weights following methionine supplementation and an immune challenge in broilers. This absence of literature is surprising given the liver's diverse roles in immune function and because the liver is the principal creator of glutathione, a potent anti-oxidant, and metabolite of methionine metabolism (Lu, 2009; Wu et al., 2004). Both primary and secondary immune organs are influenced by methionine supplementation and deficiency in chickens; however, it appears primary lymphoid organs may be more sensitive to changes in the level of methionine in the diet (Al-Mayah, 2006; Deng et al., 2007; Wu et al., 2012). Immune-induced alterations in organ weights are important to analyze when considering where methionine might provide the most support to the broiler immune system and what biological assays can be targeted to elucidate the relationship between methionine and broiler health.

The immune system is constantly developing and adapting as birds gain exposure to different pathogens and environmental stressors. A developed immune system is necessary for functionality. Early research into the relationship between methionine and immune function demonstrates that an optimized humoral response to Newcastle disease virus requires less methionine than what is required for a maximal growth response (Bhargava et al., 1970, 1971b). Contrarily, non-infectious immune challenges exhibit a more generous requirement for methionine to promote an optimal humoral response relative to that needed for the highest growth performance (Tsiagbe et al., 1987a). The immune response is also impacted by the ratio of methionine to cysteine during acute, humoral, and cellular immune challenges (Tsiagbe et al., 1987b; Takahashi et al., 1997). Specifically, cysteine is roughly 84% and 70% effective in sparing methionine for an IgG and phytohemagglutinin-P index response, respectively (Tsiagbe et al., 1987b). These early studies yield mixed results where the level of methionine needed by

the immune response was found to change based on the immune challenge and parameters evaluated.

More recent research investigates using both pathogenic and non-pathogenic immune stimuli. Birds supplemented with 1.2 or 0.9% methionine during the starter and grower phases, respectively, displayed greater HI titer and IgG 17 and 21 days post-inoculation with Newcastle disease virus compared to birds fed 0.45 and 0.33% (Mirzaaghatabar et al., 2011). Despite differences in immune activity, there was no difference in feed conversion ratio at the end of the starter or grower phase between the different levels of methionine supplementation (Mirzaaghatabar et al., 2011). Swain and Johri (2000) also made use of a Newcastle disease virus challenge in broilers fed varying levels of supplemental DL-methionine, yet when they measured antibody titers by HI and enzyme-linked immunosorbent antibody assay (ELISA) to Newcastle disease virus, they observed little difference in groups fed supplemental DL-methionine from 0 – 4.5 g/kg (3.66 – 8.70 g/kg total). Actually, the birds receiving 3.0 g/kg of supplemental DL-methionine had decreased antibody titers by HI and ELISA compared to the other supplemental levels (Swain and Johri, 2000). Despite slight differences observed for supplemental DL-methionine on the humoral response, the highest level of supplemental DL-methionine, 4.5 g/kg, produced the highest percent of leukocyte migration inhibition as a measure of the cell-mediated response (Swain and Johri, 2000). A similar finding by Rama Rao and colleagues (2003) showed a difference in cell-mediated immunity and supplemental DL-methionine, for an increased level of supplemental DL-methionine, 5.00 g/kg, was needed to produce the highest wattle thickness index in response to phytohemagglutinin-P, while feed efficiency was optimized at 3.91 g/kg of supplemental DL-methionine (Rama Rao et al., 2003). Interestingly, Rama Rao et al. (2003) reported an increase in HI titers in response to higher concentrations of supplemental DL-

methionine during immune stimulation, unlike Swain and Johri (2000), and this may be because Rama Rao et al. (2003) used a non-pathogenic challenge, sheep red blood cells, compared with a viral pathogenic challenge (Swain and Johri, 2000; Rama Rao et al., 2003). The findings from Rama Rao et al. (2003) that more supplemental methionine was needed to support the humoral immune response than what was needed to promote optimal growth align with Tsiagbe et al. (1987a), who also utilized sheep red blood cell immunization (Tsiagbe et al., 1987a; Rama Rao et al., 2003).

Using a protozoal (i.e., *Eimeria*) immune challenge in broilers to investigate the impact of methionine plus cysteine supplementation on protozoal-humoral immunity, researchers found that supplementation alone as measured by plasma anti-*Eimeria* IgG titer, intestinal luminal anti-*Eimeria* IgA titer, and total intestinal IgA production, had little impact (Ren et al., 2020). These authors bring up a crucial point to not only help describe why the humoral response during this study was not as robust as predicted but, moving forward, a consideration to take into account for amino acid requirements during immune function in broilers (Ren et al., 2020). Because the authors hypothesized that alterations in the requirement and ratios for essential amino acids might occur as a result of demand by the immune system, it warranted thought into whether or not other amino acids are impacted by the manipulation of one single amino acid in the diet (i.e., effectively enabling other amino acids to become limiting) (Ren et al., 2020). The authors highlight the importance of considering increasing other essential amino acids at a fixed level across diets, especially those with known immune implications when formulating broiler diets for requirements during immune function (Ren et al., 2020).

A paper by Rubin et al. (2007) applied a unique approach to unveiling the immune-supporting properties of methionine by creating a paradigm of immune stimuli of both

pathogenic and non-pathogenic origin throughout the study, including Marek's disease, fowl pox, infectious bronchitis, Freund's complete adjuvant, sheep red blood cells, and avian tuberculin, and supplementing the diet with methionine in the form of DL-HMTBA (Rubin et al., 2007). There was no impact of supplemental methionine on the humoral response to sheep red blood cell HI titers among the mixed immune challenge; however, the authors reported that birds receiving 0.31 and 0.29% or 0.66 and 0.64% total digestible methionine during the starter and grower phases, respectively, had decreased cellular difference (i.e., change in wattle weight following tuberculin injection) in comparison to birds receiving 0.51 and 0.49% total digestible methionine (Rubin et al., 2007). Even though the lower and higher supplementation groups exhibited decreased cellular differences than the middle supplementation group; nevertheless, a change was observed. This change demonstrates that the dietary level of methionine is influential, but to what capacity remains unknown. Concerning acquired immunity, these studies suggest the cellular and humoral responses are influenced by dietary methionine concentration, but the cell-mediated response appears to be more sensitive.

Quantifying specific humoral and cellular immune responses is a more common approach for methionine and immune function studies in broilers. Less literature is available on the acute, innate response. A TSAA deficient diet (5.6 g of sulfur amino acids/kg of diet) fed to broilers during an acute inflammatory challenge (*E. coli* lipopolysaccharide (LPS)) produced less plasma α-1-acid glycoprotein, an acute-phase protein, compared to diets supplemented with sulfur amino acids (Takahashi et al., 1997). Interestingly, addition of cysteine at 3.7 g/kg of diet to the TSAA deficient diet increased IL-1-like activity over twice as much as the TSAA deficient diet (Takahashi et al., 1997). An additional study in broiler chicks focused on the innate response following acute inflammatory stimuli and methionine supplementation. The authors concluded a

decrease in the methionine requirement for growth during acute inflammatory stress (Klasing and Barnes, 1988). Saline-injected birds had the most maximum feed efficiency at 0.7% total methionine in the diet compared to birds challenged with *E. coli* LPS or *S. aureus* attaining optimal feed efficiency at 0.5% total methionine in the diet (Klasing and Barnes, 1988). In immune challenged birds, optimal feed efficiency was achieved at a level lower than what was recorded for unchallenged broilers (Klasing and Barnes, 1988). In an additional experiment from the same paper, birds challenged with *E. coli* LPS, *S. aureus*, and *S. typhimurium* LPS and fed a methionine adequate diet (0.66% total methionine) had higher levels of plasma IL-1-like activity relative to a methionine-deficient diet (0.30% total methionine) (Klasing and Barnes, 1988). These studies demonstrate that methionine may play a vital role during an innate immune response.

Methionine supplementation to benefit immune function in broilers has been investigated, but remaining gaps include mechanisms for how this is accomplished, the magnitude of the benefit, and how the industry can utilize this knowledge. Some of these studies suggest that the requirement needed to support optimal growth may not be enough to support optimal immune function in broilers. Feeding to a birds' needs, in terms of both growth and immune function, better enables nutritionists to promote broiler health.

Methionine and immune-related compensatory gain

It is well documented that exposure to an immune challenge like a vaccine, infection from a pathogen, or heat stress can cause significant loss in growth performance in broilers (Lehman et al., 2009; Quinteiro-Filho et al., 2010; Rochell et al., 2016b). In some instances, a period of decreased growth is matched with a period of recovery, and a bird can demonstrate increased efficiency compared to healthy birds (Zubair and Leeson, 1996). In broilers, this

concept is known as compensatory growth or gain and can be observed after restriction from feed or infection (Plavnik and Hurwitz, 1991; Voeten et al., 1988). The magnitude of change in growth efficiency during the recovery period following restriction from feed can be impacted by how developed the bird is at initiation, how long and intense the restricted period is, genetics, and the nutrient content of the diet during and after restriction (Wilson and Osbourn, 1960; Zubair and Leeson, 1996). Thus, influencing the nature of compensatory growth represents a unique opportunity for dietary intervention.

During compensatory gain, the requirement for dietary amino acids may be altered, and if these requirements are not sufficiently met, the window of time for a bird to undergo an improvement in growth efficacy could be lost (Klasing and Leshchinsky, 2000). Increasing essential amino acids by 10-20% in the diet improves the feed efficiency of birds by almost 15% in the two weeks immediately following a six-day feed restriction period compared to birds not restricted and fed a control diet (Plavnik and Hurwitz, 1989). A period of compensatory gain resulting from decreased growth performance during an immune challenge can also be influenced by dietary amino acid levels. Male birds subjected to chronic summer-like temperatures for four weeks and fed lysine and methionine + cysteine at 90% of the recommended level showed optimal feed conversion; however, interestingly, when a naturally occurring viral infection of Gumboro disease (infectious bursal disease) infected half of the birds, 110% of the recommended level of lysine and methionine + cysteine was needed to attain optimal feed conversion (Kubena et al., 1972). Importantly, this shows that prolonged exposure to high temperatures alone is not enough to alter the requirement for lysine and methionine + cysteine in birds, but in combination with a disease, it can change the demand for these specific amino acids. These alterations in the nutritional requirements of amino acids from this study

demonstrate that many factors, in this case, heat exposure vs. heat exposure and disease, can impact the potency of the compensatory gain period.

The innate immune response starts at the first sign of infection. In contrast, the adaptive response is precise and takes time to develop, and therefore, may overlap with an observed period of compensatory gain. During this period, amino acid requirements must be adequate for what is needed to undergo compensatory gain and support a robust adaptive immune response, which can be long-lasting. Broilers vaccinated with a Newcastle disease virus vaccine at 25 and 50 days of age produced viral-specific antibodies (IgY) at 91 days of age, almost six weeks after the second immunization (Luo et al., 2013). Therefore, if a bird experiences a period of compensatory gain due to an immune challenge, there is potential for this to coincide with the bird's adaptive immune response to the said challenge, equating to a more considerable need for methionine.

Following exposure to a disease challenge, the compensatory growth period represents a fortunate opportunity for producers to utilize dietary intervention to better support the broiler’s ability to overcome infection and restore homeostasis. Such interventions can even be tailored to the specific disease present in their barn. There is little research on methionine's requirement following an immune-induced period of compensatory gain and how this amino acid impacts the bird's ability to develop immunological memory via the adaptive response. Further research is warranted to determine whether nutritional intervention during the recovery phase via supplementation of methionine after an immune challenge can aid in restoring homeostasis in broilers efficiently by offering additional resources to the recovering immune system.

Conclusion

The amino acid methionine serves many biological purposes for a bird. Its routine inclusion into broiler diets because of its known limiting nature has made it the scrutiny of much poultry science nutrition literature; however, the body of literature lacks estimation of the requirement for methionine under varied environmental rearing scenarios, specifically immune challenges. The diverse utilization of methionine includes but is not limited to biological methylation, formation of cysteine and the potent anti-oxidants glutathione and taurine, polyamine synthesis, pyruvate formation, and protein synthesis. Because some of the known uses of methionine have immunological implications, it permits further thought about whether the requirement for methionine during an immune challenge is altered. To elucidate the relationship between broiler health and methionine, investigating the requirement of methionine in broilers during non-challenged and immune challenged conditions and the subsequent impact on growth and immune parameters will be assessed herein.

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CHAPTER 2: METHIONINE REQUIREMENT OF BROILERS FED SEMI-PRACTICAL CORN-SOYBEAN MEAL-BASED DIETS

Abstract: Two dose-titration experiments were conducted to evaluate 2 Met sources, DL-Met and DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA), in a diet formulation that permits manipulation of dietary Met and cysteine levels to determine the requirement of Met, bioefficacy of Met sources, and impact to organ growth and development. For Experiment 1, 7 low-CP dietary treatments were fed during the grower phase (d 10 – 24): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). For Experiment 2, 12 low-CP dietary treatments were fed to broilers during the grower phase (d 10 – 24): LTSAA, Met0, and Met0 supplemented with graded DL-Met (0.072% increments; Met1 – Met5) or DL-HMTBA (0.103% increments; HMTBA1 – HMTBA5). Growth performance was increased ($P < 0.001$) with Met supplementation. The LTSAA and Met0 differed for growth performance during d 10 – 24, but only during Experiment 2. Absolute and relative gastrointestinal and immune organ weights and lengths were influenced by Met supplementation ($P <0.05$). Relative liver weight decreased ($P < 0.001$) in diets supplemented with at least Met2 or HMTBA3 and above compared to diets devoid of Met supplementation. From the fitted 1-slope broken-line regression analysis, the estimated requirement of supplemental DL-Met ranged from 0.18% – 0.19% (0.33% – 0.34% total Met) for BW gain and 0.12% – 0.15% (0.27% – 0.30% total Met) for gain:feed. Estimated requirements for supplemental DL-HMTBA were 0.22% (plus 0.15% Met) for BW gain and 0.17% (plus 0.15% Met) for gain:feed. The bioefficacy of DL-HMTBA relative to DL-Met was estimated to be 78% for BW gain and 82% for gain:feed. These data demonstrate that an adequate level of dietary Met is required for optimal growth, and inclusion of dietary L-

cystine at 0.36% (0.51% total cysteine) to a Met deficient low-CP diet decreases growth. Additionally, it suggests DL-HMTBA is less bioefficacious than DL-Met for growth, and Met supplementation promotes liver efficacy.

Introduction

There are complex biological relationships between nutrition and metabolism, and the immune system. Prominent examples of this are the reductions in feed intake following infection and subsequent decrease to growth (Tan et al., 2014; Rochell et al., 2016b). However, detailed understandings of how individual amino acids play into these interactions are understudied. In birds, the requirement of an amino acid is dynamic and is affected by the breed and purpose of the bird (e.g., breeder vs. layer vs. broiler vs. dual-type) and varies based on genetics, sex, and age. In addition, the physiological state of the bird, like whether they are undergoing growth, reproducing, activating the immune system, or maintaining body weight, can alter amino acid metabolism. For broilers, an amino acid requirement is usually expressed in terms of growth, such as BW gain and feed efficiency, which reflects the metabolic cost of an amino acid needed for both maintenance and growth. Although these requirements promote optimal growth, they may differ from what is required for optimal function of other biological systems, like the immune system, and change when parameters other than growth are assessed. Supplementing essential amino acids with known immune involvement, such as Arg, Trp, and Thr improves the broiler immune response (Ruiz-Feria and Abdukalykova, 2009; Emadi et al., 2011; Sigolo et al., 2017). Resources utilized by poultry nutritionists that report the minimum required level of an amino acid have been mainly estimated from broilers reared in unchallenged conditions and are primarily based on maximal growth outcomes. Therefore, studies that identify amino acid

requirements in challenged conditions may better align with industry settings and reduce nutrient wastages from the diet.

Protein and amino acid nutrition is the most vital component of producing lean muscle protein, the main saleable product of meat-type broiler production. In most standard and low crude protein corn soybean-meal-based broiler diets, Met is the first (i.e., most) limiting amino acid for protein synthesis in terms of growth and, therefore, nearly always supplemented to the diet in combination with intact protein sources (Edmonds et al., 1985; Fernandez et al., 1994). The amino acid Met is used in many cellular activities, including methylation, sulfur donation, pyruvate production, formylmethionine formation, and is a precursor to cysteine and the potent anti-oxidants taurine and glutathione (Brosnan and Brosnan, 2006). Specific to immunity, Met serves a role in the biosynthesis of polyamines, which can influence immunity themselves and facilitate cell growth (Handa et al., 2018; Hesterberg et al., 2018; Igarashi and Kashiwagi, 2000). Additionally, methylation of DNA regulates gene transcriptional activity of immune functions like proliferation, differentiation, and regulation of T and B cells, mediation of NK cells, production of pro-inflammatory cytokines, and complement activation (Suarez-Alvarez et al., 2012).

The requirement for Met is often expressed as Met + cysteine because of Met's ability to furnish cysteine, and the level at which it is supplied to the diet is meant to satisfy the need for both Met and cysteine. Feeding Met alone to satisfy the requirement for Met + cysteine yields a higher minimum requirement for Met than meeting the requirement with both supplemental Met and cysteine due to the unequal metabolic conversion of Met to cysteine on a molar basis (Baker and Dilger, 2009). Indeed, the addition of supplemental cysteine to the diet effectively spares the use of methionine, but its addition can also change the extent of the growth response (Baker and

Dilger, 2009). Because of these factors, it is essential to consider the level of cysteine when determining the requirement for methionine.

Two commonly used sources of supplemental Met in broiler diets are DL-Met and DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA). The enzymes needed for metabolism to L-Met, the form considered 100% useable by cells, differs between the 2 sources, although they share a common intermediate, 2-keto-4-(methylthio) butanoic acid (Dibner, 2003). The amount of a source, DL-Met or DL-HMTBA, found in a commercially available product can vary (Vázquez-Añón et al., 2006). In addition, the bioavailability (i.e., bioefficacy) between sources, or the amount digested, absorbed, and utilized by the animal, has been reported to be dissimilar in terms of growth performance (Littell et al., 1997; Sauer et al., 2008). It is important to consider the bioavailability of sources when estimating the requirement of Met. Ingestion of a Met source by the bird does not necessarily mean it is available for protein synthesis or other metabolic uses. If a Met source has a lowered bioavailability, it may artificially inflate the requirement of Met if left unaccounted for.

A method to estimate an amino acid requirement for a variable that elicits a dose-response is by 1-slope broken-line regression modeling (Robbins et al., 1979, 2006). Therefore, the objective of Experiment 1 was to investigate if dietary treatments of graded levels of supplemental DL-Met elicit a dose-response in terms of growth for broilers during the grower phase (d 10 – 24), allow for the estimation of the requirement of supplemental DL-Met for maximal growth, and impact the absolute and relative weights and lengths of gastrointestinal and immunological organs. Because DL-Met is considered 100% efficacious relative to L-Met, DL-Met was chosen as the Met source for experiment 1 (Dilger and Baker, 2007a). The objective of Experiment 2 was the same as Experiment 1 but included an additional source of Met, DL-

HMTBA, and sought to estimate the bioefficacy of DL-HMTBA relative to DL-Met in terms of growth. We hypothesized that broilers consuming diets with higher levels of supplemental Met, regardless of source, would have increased growth performance, increased absolute weights and lengths of organs, and decreased relative weights and lengths of organs compared to birds fed diets low or devoid of supplemental Met. The estimated requirements determined during these experiments are specific to our rearing facility, genetics, and dietary profile and will set the foundation for future data chapters' experimental designs.

Materials and Methods

All animal husbandry and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois Urbana-Champaign before initiating experiments.

General Bird Husbandry

In both experiments, Ross 308 male broiler chicks were obtained from a commercial hatchery (Hoovers Hatchery, Rudd, IA) and transported to the University of Illinois before being placed in thermostatically controlled battery cages measuring 99 cm long, 66 cm wide, and 33 cm high (model SB5T; Alternative Design Manufacturing, Siloam Springs, AR) with raised-wire flooring. The rooms housing the battery cages were environmentally controlled according to breeder standards and included continuous lighting. Upon arrival, chicks were placed in battery cages with access to water overnight. Following an overnight fast, at study d 0, chicks were individually weighed, wing-banded, allotted according to initial BW, and randomly assigned to individual treatment groups. From study d 0 to 10, all chicks were fed a practical corn-soybean meal-based starter diet (**Table 2.1**). On study d 10, all birds were switched to their respective experimental grower diets (**Table 2.2**). All diets were fed in the mash form and provided *ad*

libitum in a trough feeder (61 cm long, 9 cm wide, and 9 cm deep), in addition to tap water via 2 industry-standard nipple drinkers. Experimental grower diets were analyzed by AMINO-Lab (Evonik Product Quality & Regulatory Affairs, Hanau, Germany) for crude protein (Association of Official Analytical Chemists (AOAC) method 990.03; AOAC International, 2000) and amino acids (AOAC method 994.12; AOAC International, 2000) using an amino acid analyzer (Biochrom30+; Cambridge, UK) (**Tables 2.3 & 2.4**). Analyzed composition values were similar to the calculated composition values.

On study d 24, all birds were euthanized via CO_2 asphyxiation, and 1 bird per pen (randomly chosen) was used for quantifying the absolute weight (g) of the following unflushed organs: duodenum, jejunum, ileum, ceca, colon, spleen, liver, bursa, gizzard, proventriculus, crop, and pancreas. The same bird was also used for measuring the absolute length (cm) of the following gastrointestinal organs: duodenum, jejunum, ileum, ceca, and colon. Organ weights and lengths relative to BW or total tract length were calculated from the captured absolute organ weights and lengths. Relative organ weight was calculated by taking the weight of the organ (g), dividing it by the live BW of the bird (g), and multiplying by 100, while the relative length was calculated by taking the length of the organ (g), dividing it by the total tract length (cm), and multiplying by 100. Total tract length was calculated by adding the absolute length (cm) of the duodenum, jejunum, and ileum. Individual chick weights and pen feeder weights were captured on study d 0, 10, and 24 to calculate BW gain, feed intake, and gain:feed.

Experiment 1

A total of 294 day-old Ross 308 male broiler chicks (42 chicks/treatment) were obtained for utilization in an experiment with 7 treatments, 7 replicates per treatment, and 6 chicks per replicate. The 7 low-CP dietary treatments were fed during the grower phase (d 10 – 24) and

included a low total sulfur amino acid diet (LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), and Met0 with supplemented graded levels of DL-Met (0.072% increments; Met1 – Met5). The supplemental levels were chosen such that the LTSAA and Met0 diets were formulated to be devoid of supplemental DL-Met and contain 29% of the total Met requirement compared to the Ross Broiler: Nutrient Specifications (2019) for as-hatched broilers grown to 3.60 – 4.00 kg of BW for the grower feeding phase (d 11 – 24), while Met5, the diet containing the highest level of supplemental DL-Met, was formulated to contain 100% of the total Met requirement (Aviagen, 2019a). Compared to the Poultry NRC (1994) for broilers aged 0 – 3 wk, LTSAA and Met0 were formulated to 29% of the total Met requirement, while Met5 was formulated to 101% of the total Met requirement (NRC, 1994). Because the level of dietary cysteine can change the requirement of Met due to its sparing capacity on Met, dietary cysteine was formulated to be fed in slight excess and at an equal level in all dietary treatments used for requirement estimations (i.e., all but LTSAA) (Baker and Dilger, 2009). Supplemental DL-Met (MetAMINO 99%, Evonik Nutrition and Care GmbH, Hanau, Germany) and L-cystine were added to the diet at the expense of dextrose, while the remainder of ingredients were kept constant across treatments. Amino acids aside from DL-Met and L-cystine were fed adequately and formulated using requirements procured from AMINOChick 2.0 (Evonik Nutrition and Care GmbH, Hanau, Germany). The BW gain and gain:feed calculated for the grower phase d 10 – 24 were utilized to estimate supplemental DL-Met requirements.

Experiment 2

A total of 504 day-old Ross 308 male broiler chicks (42 chicks/treatment) were obtained for utilization in a study with 12 treatments, 7 replicate cages per treatment, and 6 chicks per replicate cage. The 12 low-CP dietary treatments were fed during the grower phase (d 10 – 24)

and included LTSAA, Met0, and Met0 supplemented with graded DL-Met (0.072% increments; Met1 – Met5) or DL-HMTBA (0.103% increments; HMTBA1 – HMTBA5). Dietary cysteine was formulated to be fed in slight excess and at an equal level in all dietary treatments used for requirement estimations similar to Experiment 1. Supplemental DL-Met (MetAMINO 99%, Evonik Nutrition and Care GmbH, Hanau, Germany) and L-cystine were added to the diet at the expense of dextrose. Supplemental DL-HMTBA was also added to the diets at the expense of dextrose and was accomplished using a silica carrier (SIPERNAT specialty silica, Evonik Nutrition and Care GmbH, Hanau, Germany) that contained 58.52% DL-HMTBA and 41.48% carrier + water. To ensure consistency across treatments, the silica carrier alone was also added to diets LTSAA and Met0 – Met5 at inclusion levels similar to that found in diets HMTBA1 – HMTBA5 at the expense of dextrose. The remainder of the ingredients were formulated to be consistent across treatments. The levels of supplemental Met source were chosen based on the results garnered in Experiment 1. Supplemental DL-HMTBA was assumed to be 70% bioefficacious on an equimolar basis in relation to DL-Met for growth for formulation purposes. Thus, diets Met1-Met5 and HMTBA1-HMTBA5 were formulated such that birds would grow similarly. The requirement for supplemental DL-Met and DL-HMTBA for BW gain and gain:feed and the bioavailability for DL-HMTBA in relation to DL-Met for BW gain and gain:feed were estimated for the grower period (d 10 – 24).

Statistical Analysis

The experimental design for each experiment was a completely randomized design. All analyses were an ANOVA using the MIXED procedure of SAS (version 9.4; SAS Institute, Cary, NC) to determine whether the model was significant. When appropriate, means separation was conducted. Individual cage served as the experimental unit, and dietary treatment was

considered the main effect of the model, while the replicate cage was included as a random effect. In all instances, outliers were identified as having an absolute Studentized residual value of 3 or greater, and significance was accepted at a *P*-value of less than 0.05. The requirement of supplemental DL-Met or DL-HMTBA was estimated using a 1-slope broken-line regression model (Robbins et al., 1979, 2006). The LTSAA diet was removed from all analyses used to estimate the requirement or bioavailability of a Met source. The bioavailability of a Met source was estimated using a nonlinear, exponential regression model (Littell et al., 1997).

Results

Experiment 1

The growth performance results from Experiment 1 are displayed in **Table 2.5**. There were no significant differences between treatment groups for BW gain, feed intake, or gain:feed during the starter phase. Birds receiving the highest supplemental level of DL-Met during the grower phase, Met5, had the highest ($P < 0.001$) BW gain from d 10 – 24 and 0 – 24 compared to all other treatment groups. Feed intake also increased ($P < 0.001$) the most by the highest level of supplemental DL-Met (Met5) during d 10 – 24 and 0 – 28, although Met5 was not significantly different from Met3 or Met4. From d 10 – 24 and 0 – 24, gain:feed was increased ($P < 0.001$) in birds receiving diets Met3 – Met5. The LTSAA and Met0 birds were not significantly different ($P > 0.05$) from each other for BW gain, feed intake, and gain:feed from d 10 – 24 and 0 – 24.

The BW gain and gain:feed data excluding the LTSAA diet were fitted to a 1-slope broken-line regression model to estimate the requirement of supplemental DL-Met during the grower phase of d 10 – 24 (**Table 2.6**). The requirement of supplemental DL-Met for BW gain

and gain:feed were estimated to be 0.18% (0.33% total Met) of the diet to reach a level of 780 g/bird and 0.15% (0.30% total Met) of the diet to reach a level of 641 g/kg, respectively.

Dietary treatments during the grower phase influenced ($P < 0.001$) the absolute weight of all organs at d 24 (**Table 2.7**). The relative weights of the spleen and bursa at d 24 were not influenced ($P > 0.05$) by the level of supplemental DL-Met in the diet during the grower phase (**Table 2.8**). Birds receiving diets Met2 or above regarding graded DL-Met level had decreased ($P <0.001$) relative liver weights.

The impact of dietary treatment on the absolute and relative length of GIT organs at d 24 is reported in **Table 2.9**. All absolute lengths of organs and total tract length were impacted ($P < 0.001$) by dietary treatment at d 24. The relative length of the duodenum decreased ($P <0.001$) in Met4 and Met5 birds but was not significantly different from Met2 and Met3 birds. The relative length of the jejunum was not influenced ($P = 0.295$) by dietary treatment. The relative length of the ileum was increased ($P < 0.001$) in Met4 birds but was not significantly different from Met2 and Met5 birds.

Experiment 2

The growth performance results from Experiment 2 are displayed in **Table 2.10**. The BW gain, feed intake, and gain:feed was not significantly different ($P > 0.05$) among treatments during the starter phase d 0 – 10. During d 10 – 24 and 0 – 24, Met4 birds had increased ($P <0.001$) BW gain and were not significantly different from Met3, Met5, HMTBA3, and HMTBA4. Feed intake was increased ($P <0.001$) during d 10 – 24 for Met3 and Met4, but not significantly different than Met5, HMTBA3, HMTBA4, and HMTBA5. Throughout the entire trial (d 0 – 24), dietary treatment Met3 was increased ($P <0.001$) for feed intake, but not significantly different from Met4, Met5, HMTBA3, and HMTBA4. The gain:feed from d 10 – 24

was increased (P <0.001) for HMTBA3 birds compared to the other treatment groups, although not significantly different from Met2, Met4, Met5, HMTBA2, HMTBA4, and HMTBA5. The HMTBA3 with the addition of Met4 and Met5 birds had increased (P < 0.001) gain:feed from d 0 – 24 and were not significantly different from Met2, HMTBA2, HMTBA4, and HMTBA5 birds. From d 10 – 24, Met0 birds had decreased (P <0.001) BW gain, feed intake, and gain:feed compared to the LTSAA birds; however, from d 0 – 24, Met0 and LTSAA birds did not significantly differ for BW gain, feed intake, and gain:feed.

The BW gain and gain:feed data excluding the LTSAA diet were fitted to a 1-slope broken-line regression to estimate the requirement of supplemental DL-Met or DL-HMTBA during the grower phase d 10 – 24 (**Table 2.6**). The requirement of supplemental DL-Met for BW gain and gain:feed were estimated to be 0.19% (0.34% total Met) of the diet to reach a level of 801 g/bird and 0.12% (0.27% total Met) of the diet to reach a level of 605 g/kg, respectively. The requirement of supplemental DL-HMTBA for BW gain and gain:feed were estimated to be 0.22% (plus 0.15% total Met) of the diet to reach a level of 778 g/bird and 0.17% (plus 0.15% total Met) of the diet to reach a level of 612 g/kg, respectively.

The bioavailability of DL-HMTBA in relation to DL-Met was estimated during the grower phase d 10 – 24 for BW gain and gain:feed by fitting the data excluding the LTSAA diet to a nonlinear exponential regression analysis (**Figures 2.1 & 2.2**). The bioavailability of supplemental DL-HMTBA relative to DL-Met was estimated to be 78.4% for BW gain and 81.7% for gain:feed.

The absolute weight of organs at d 24 is reported in **Table 2.11**. Eleven out of the 12 organs were impacted (P <0.001) by the experimental dietary treatments. The relative weight of organs at d 24 is reported in **Table 2.12**. Except for the spleen and bursa, all relative organ

weights were impacted (P <0.043) by experimental dietary treatments. The relative weight of the liver was lowered (P <0.001) by Met5 compared to the other treatment groups and was not significantly different from HMTBA3 and HMTBA4 birds.

The absolute and relative lengths of organs at d 24 are displayed in **Table 2.13**. Dietary treatments impacted (P < 0.005) the absolute length of all measured GIT organs and the total tract length. No difference (P > 0.05) was found between treatment groups for the relative lengths of the duodenum, jejunum, and ileum.

Discussion

In these experiments, we sought to validate a dietary profile that permits manipulation of dietary Met and cysteine levels using two sources of Met, which is essential for determining the Met requirement of broilers in both healthy and immune stimulated conditions. Moreover, the dietary profile also needs to allow for the comparison of bioefficacy of Met sources. To determine the minimal requirement using a 1-slope broken-line regression model, a dose-response is required from the assessed independent variable (Robbins et al., 1979, 2006). The bioefficacy of Met sources can be estimated from non-linearized data using a nonlinear regression model (Littell et al., 1997). We observed, regardless of dietary Met source, that the broiler growth response is improved when supplemental Met is added to the diet (**Table 2.5 & 2.10**). However, the addition of L-cystine to a total sulfur amino acid deficient diet reduced the broiler growth response (**Table 2.10**). In addition, the investigated graded levels of supplemental Met adequately fit the 1-slope broken-line model and granted the estimation of the bioefficacy of DL-HMTBA relative to DL-Met. Our findings suggest DL-HMTBA is less bioefficicacious than DL-Met in terms of growth. Methionine supplementation, regardless of source, also impacted gastrointestinal and immune organ metrics, such that birds fed Met supplemented diets generally

had increased absolute organ weights and lengths, decreased relative organ weights, and marginally impacted relative organ lengths.

In Experiment 1, graded dietary concentrations of supplemental DL-Met during the grower phase elicited improvements in growth performance, as expected. The growth response from birds during the experimental grower phase was indeed positive; however, the maximum BW gain and gain:feed reported in Experiment 1 were less than the respective breeder performance objectives. The BW gain (1020 g/bird) of birds throughout the experiment was maximized for Met5 birds. Compared to Ross 308 Performance Objectives for males d 0 – 24, this value is about 83% of the reported BW gain (1232 g/bird) (Aviagen, 2019b). The Met3 birds had the highest gain:feed (678 g/kg) from d 0 – 24, which is roughly 88% of the gain:feed value (769 g/kg) reported in Ross 308 Performance Objectives for males d 0 – 24 (Aviagen, 2019b). Not only were growth performance metrics lower than breeder specifications in Experiment 1, but the estimated requirements for total Met from the 1-slope broken-line regression analysis were also lower. Compared to the requirement for total Met from breeder specifications (as-hatched broilers grown to 3.60 – 4.00 kg of BW for d 11 – 24) and the 1994 Poultry NRC (broilers age 0 – 3 weeks), our estimated requirement for BW gain was only 65% and 66%, respectively, and for gain:feed, roughly 59% and 60%, respectively, of the reported requirements (NRC, 1994; Aviagen, 2019a). Overall, broilers had increased growth performance with the addition of dietary Met but required less total Met to achieve optimal BW gain and gain:feed and failed to reach performance objectives by as much as 17%.

Plausible explanations for why our estimated values were less than the reported requirements and performance objectives from the previously mentioned documents could be differences in rearing conditions, specifically battery vs. floor-pen settings. Previous experiments

conducted in batteries from our lab yield growth performance metrics that more closely reflect those captured in Experiment 1 (Rochell et al., 2016b; Oelschlager et al., 2019). In an experiment from our lab where broilers were raised in batteries, a diet containing 9.8% CP (same as Experiment 1) from corn and soybean meal supplemented with 18 individual amino acids elicited similar BW gain and feed efficiency compared to a control diet (i.e., industry standard) containing 18.4% CP from corn and soybean meal and supplemented with 4 individual amino acids (Rochell et al., 2016a). Another cause for differences could be the dietary profile. The estimates of requirements and performance objectives were likely calculated from broilers fed practical diets. Broilers fed purified crystalline amino acid diets supplemented with graded concentrations of L-Met, and adequate cysteine required 0.24% total Met for BW gain from d 8 – 17 (Dilger and Baker, 2007b). Despite unequal growth periods, 0.24% total Met is 48% of what is required by the 1994 Poultry NRC for broilers age 0 – 3 weeks (NRC, 1994; Dilger and Baker, 2007b). The diet utilized in Experiment 1 was semi-practical (i.e., semi-purified) and had a reduced total Met requirement (0.33%) compared to the NRC (0.50%), but less than a wholly purified diet. Additionally, the level of dietary cysteine influences the requirement for Met and the subsequent growth response (Dilger and Baker, 2007a). When feeding Met alone to satisfy the requirement for Met + cysteine, not only is a higher requirement for Met needed but the growth response to supplemental Met is decreased by almost half compared to meeting the requirement with Met and excess dietary cysteine (Dilger and Baker, 2007a; Baker and Dilger, 2009). Because the diets fitted to the 1-slope broken-line regression model for estimating supplemental Met also contained calculated dietary cysteine in excess, the requirement of total Met would be expected to be less than when practical diets are used for estimations.

Despite differences in the requirement for Met and growth metrics compared to industry standards, the 1-slope broken-line regression model fitted to the data to estimate the requirement for supplemental DL-Met for BW gain and feed efficiency had calculated R^2 values of at least 0.98 or above, signifying that our graded levels of dietary Met elicited response variables that fit the data well. In addition, individual replicates, rather than overall mean values per dietary treatment, were utilized in the regression analysis for estimating the requirements of supplemental Met. The use of overall mean values can reduce the residual sum-of-squares, which artificially inflates the R^2 value (Motulsky and Christopoulos, 2003). Because the graded levels of supplemental DL-Met adequately fit the model, the second Experiment was designed similar to the first regarding levels of available Met and included the second Met source, DL-HMTBA.

Similar to growth performance in Experiment 1, supplementation of Met to the diet, regardless of source, improved growth performance in Experiment 2. Additionally, the estimated requirements for supplemental Met determined by the 1-slope broken-line regression analysis yielded similar values to Experiment 1. We observed an increased estimated requirement of supplemental DL-HMTBA than the amount of supplemental DL-Met required for BW gain (~16% increase) and gain:feed (~42% increase). Despite these differences, the requirement fell between the same fixed supplementation levels (i.e., means) of supplemental DL-Met and DL-HMTBA because we accounted for a difference in bioefficacy of DL-HMTBA relative to DL-Met during diet formulation. Estimation of the bioefficacy of DL-HMTBA relative to DL-Met can vary based on a statistical approach, the profile of the basal diet, the product type of DL-HMTBA, and the parameter being assessed (EFSA FEEDAP Panel, 2012). Because different factors can influence bioefficacy, it is essential to estimate the relative bioefficacy of DL-HMTBA to DL-Met under specific rearing conditions and dietary treatments.

An abundance of bioefficacy studies comparing DL-HMTBA to DL-Met exist in the literature, but there remains a discrepancy regarding the bioefficient value of DL-HMTBA relative to DL-Met. A multiple regression analysis by Vázquez-Añón et al. (2006) determined DL-HMTBA and DL-Met have similar bioefficacy values while a meta-analysis by Sauer et al. (2008) found different bioefficacy values for growth performance (Vázquez-Añón et al., 2006; Sauer et al., 2008). The meta-analysis by Sauer et al. (2008) analyzed 46 dose-response broiler studies derived from 27 peer-reviewed papers and estimated a bioefficacy value for DL-HMTBA relative to DL-Met to be 81% for average daily gain and 79% for gain:feed (Sauer et al., 2008; EFSA FEEDAP Panel, 2018). The bioefficacy of DL-HMTBA-Ca, the calcium salt form of DL-HMTBA commercially available to poultry producers, also differs and is estimated to be about 66% and 64% for BW gain and gain:feed, respectively (Elwert et al., 2008). A lowered bioefficacy of DL-HMTBA in relation to DL-Met is likely due to the increased number of polymers found in commercially available DL-HMTBA products, which tend to have a lower bioefficacy, and increased competition between host small intestine gut microbiota for DL-HMTBA compared to DL-Met (EFSA FEEDAP Panel, 2018). Our estimated bioefficacy values obtained from Experiment 2 align more closely with those who found DL-HMTBA to be lower in bioefficacy than DL-Met (Elwert et al., 2008; Sauer et al., 2008). The estimated bioefficacy levels from Experiment 2 were calculated using only growth response parameters. Far less literature exists that takes into consideration immune response parameters under challenged conditions. Analysis of immune response parameters or a combination of growth and immune response measurements may also change the bioefficacy value between DL-HMTBA and DL-Met.

Interestingly, during Experiment 2 only, the addition of L-cystine at 0.36% (0.51% total cysteine) of the diet (Met0) decreased BW gain, feed intake, and gain:feed compared to the LTSAA birds. A purified diet deficient in Met (0.12% total Met) supplemented with 0.35% cysteine (0.40% total cysteine) increased BW gain by over 20 grams and increased gain:feed by over 50% from d 8 – 17 posthatch (Dilger and Baker, 2007b). Experiment 2 had similar supplementation levels of L-cystine (0.35% vs. 0.36%) as those used by Dilger and Baker (2007b); however, the level of total cysteine found in our Met0 (0.51%) was more than 20% the level (0.40%) used by Dilger and Baker (2007b), due to a higher level of cysteine in the LTSAA than their basal diet (Dilger and Baker, 2007b). An additional study by Dilger and Baker (2007a) utilizing the same purified Met deficient diet (0.12% total Met) and growing period d 8 – 17, also displayed improved growth performance with the addition of L-cystine, but there were linear decreases in BW gain and gain:feed at L-cystine supplementation levels of 0.07% – 0.35% (0.12% – 0.40% total cysteine) (Dilger and Baker, 2007a). Because of the linear decreases to growth performance, it is possible that if a higher supplementation level were to have been assessed by Dilger and Baker (2007a) closer to the level utilized in Experiment 2, they would have witnessed a decrease in growth performance due to high dietary cysteine supplementation (Dilger and Baker, 2007a). The addition of L-cystine to purified diets can impede chick growth, but decreased growth is not usually witnessed until the dietary level of cysteine is well over the requirement, sometimes by as much as 7.5 times (Dilger et al., 2007). Compared to the Ross broilers nutrient requirements, the total cysteine level found in Met0 of Experiment 2 was only 1.06 times the required amount (Aviagen, 2019a). It should be brought to attention that in both of the experiments by Dilger and Baker (2007a; b) and Experiment 2, the diets were deficient in Met (Dilger and Baker, 2007a; b). Cysteine cannot be converted to Met, so the metabolic

imbalance of total sulfur amino acids in the Met0 diet may have exacerbated the bird and hampered growth. The decrease in growth by Met0 was ameliorated with the addition of supplemental Met to the diet even at the lowest level of Met1 or HMTBA1, which aligns with the explanation of an imbalance of total sulfur amino acids thwarting growth.

The absolute and relative weight of immune and gastrointestinal organs are quantified to assess organ growth, development, and efficacy (Palo et al., 1995; Bartlett and Smith, 2003; Kautzman et al., 2017; Shinde Tamboli et al., 2017; Jiménez-Moreno et al., 2019). The deficiency of an essential amino acid not only impedes growth performance but can impair relative immune organ development (Konashi et al., 2000). In Experiments 1 and 2, the absolute weights of the immune-related organs, spleen, liver, and bursa, were all significantly impacted by dietary treatment at study conclusion d 24; however, when put on a relative to BW basis, only the liver remained significantly altered by dietary Met treatment among those organs. Generally, diets devoid of Met supplementation, LTSAA and Met0, increased relative liver weight while adding Met to the diet decreased the relative liver weight. These results are similar to those found by Garcia Neto and colleagues (2000), where Met supplementation compared to no supplementation decreased the relative liver weight (Garcia Neto et al., 2000). The liver is a multi-functional organ with both metabolic and immunological implications, including processing antigens, producing acute-phase, anti-microbial, and complement proteins, and is the site of most sulfur amino acid metabolism (Mato et al., 2013; Robinson et al., 2016). Despite increasing the hepatic amino acid load of the liver and growth rate by increasing dietary inclusion of sulfur amino acids, we demonstrated the liver's ability to maintain functionality and improve efficiency.

The duodenum, jejunum, and ileum comprise the broiler small intestine, and its primary function is to digest and absorb nutrients from the diet (Svihus, 2014). Components of a diet can influence the lengths of the individual sections of the small intestine. Dietary carbohydrate sources rich in fiber, particularly non-starch polysaccharides, can reduce growth performance and increase lengths of the gastrointestinal tract (Sadeghi et al., 2015). Non-starch polysaccharides and other fiber sources possess anti-nutritional qualities, like increasing gastrointestinal viscosity, which can lower nutrient digestibility (Langhout et al., 2000). Because birds eat to satisfy their physiological requirements, increasing the length of the gastrointestinal tract following consumption of diets high in fiber compensates for impacts by anti-nutritional factors by increasing the surface area and likelihood of a nutrient being digested and absorbed. Indeed, the inclusion of supplemental Met generally increased the total tract lengths of the small intestine, but with it came improvements to growth performance. The jejunum is the section of the gastrointestinal tract where a majority of dietary protein is absorbed (Svihus, 2014). On a relative basis to total tract length, the jejunum was not influenced by dietary treatment. Broilers supplemented with Met had improved growth performance and longer absolute intestinal tract lengths. However, because minor changes to relative lengths were noted, it was concluded that supplemental Met has a marginal impact on intestinal tract length and may be more influential to growth post-absorption.

In conclusion, it was determined from these experiments that supplementation of Met and cysteine to a deficient total sulfur amino acid diet promotes growth performance and some gastrointestinal and immunological organ growth and development. Supplementation of dietary L-cystine alone to a Met and cysteine deficient diet can impede growth, likely due to an imbalance in sulfur amino acid metabolism that can disrupt homeostasis. The tested treatment

levels of supplemental DL-Met and DL-HMTBA successfully fit the model ($R^2 \geq 95$) for determining the requirement of supplemental Met based on growth outcomes. Additionally, the data adequately fit the model ($R^2 \geq 88$) for determining the bioefficacy of DL-HMTBA relative to DL-Met. Taken together, these experiments demonstrate the use of a diet formulation that allows for manipulation of both Met and cysteine levels as necessary for determining amino acid requirements in broilers in healthy conditions and permits comparison of the bioefficacy of Met sources. Whether the requirement for Met and bioefficacy of Met sources are altered during adverse conditions, like an immune challenge, is yet to be understood. Further research towards quantifying the relationship between dietary Met and immune stress on growth and immune outcomes may support better broiler health that aligns more closely with industry rearing standards.

Tables and Figures

Table 2.1. Ingredient composition of starter (d 0 – 10) diets fed to broilers in Experiments 1 and 2[1]

Item, % as-fed	Experiment 1	Experiment 2
Corn	50.09	50.14
Soybean meal	40.88	40.88
Soy oil	4.00	4.00
Sodium chloride	0.40	0.40
Limestone	1.20	1.20
Dicalcium phosphate	2.10	2.10
Vitamin premix[2]	0.20	0.20
Mineral premix[3]	0.15	0.15
Choline chloride	0.32	0.32
L-Lys HCl	0.17	0.16
DL-Met	0.38	0.37
L-Thr	0.11	0.09
Calculated composition		
AME_n, kcal/kg	2970	2979
CP	22.17	22.63
Lys	1.44	1.46
Met + Cys	1.04	1.03
Thr	0.97	0.97
Ca	1.06	1.06
Non-phytate P	0.49	0.50
Analyzed composition		
CP	22.85	23.85
Lys	1.38	1.36
Met + Cys	1.04	0.97
Thr	0.94	0.92

[1]Abbreviations: AME_n, nitrogen-corrected apparent metabolizable energy.
[2]Provided per kilogram of complete diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 μg; dl-α-tocopheryl acetate, 11 IU; vitamin B12, 0.01 mg; riboflavin, 4.41 mg; d-Ca-pantothenate, 10 mg; niacin, 22 mg; and menadione sodium bisulfite, 2.33 mg
[3]Provided as milligrams per kilogram of complete diet: Mn, 75 from MnO; Fe, 75 from $FeSO_4 \cdot 7H_2O$; Zn, 75 from ZnO; Cu, 5 from $CuSO_4 \cdot 5H_2O$; I, 0.75 from ethylene diamine dihydroiodide; and Se, 0.1 from Na_2SeO_3.

Table 2.2. Ingredient composition of the experimental grower (d 10- 24) basal diets fed to broilers in Experiment 1 and 2

Ingredient, % as-fed	Experiment 1[1]	Experiment 2[2]
Corn	31.84	32.01
Soybean meal	16.69	16.77
Dextrose	30.89	30.54
Cellulose	4.03	4.05
Soybean oil	3.53	3.54
Sodium bicarbonate	0.50	0.51
Limestone	1.04	1.04
Dicalcium phosphate	2.19	2.20
Vitamin premix[3]	0.20	0.20
Mineral premix[4]	0.15	0.15
Choline chloride	0.37	0.37
Potassium carbonate	0.78	0.79
Amino acid mixture	7.80[5]	7.82[6]

[1]For Experiment 1, supplemental DL-Met and L-cystine were substituted for dextrose to create the experimental dietary treatments Met0 – Met5. Additional dextrose was added to the basal diet at 0.72% to create the LTSAA diet.
[2]For Experiment 2, supplemental DL-Met, DL-HMTBA + Sipernat, Sipernat, and L-cystine were substituted for dextrose to create the experimental dietary treatments Met0 – Met5 and HMTBA1 – HMTBA5. Additional dextrose was added at 1.18% and Sipernat at 0.06% to the basal diet to create the LTSAA diet.
[3]Provided per kilogram of complete diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 μg; dl-α-tocopheryl acetate, 11 IU; vitamin B12, 0.01 mg; riboflavin, 4.41 mg; d-Ca-pantothenate, 10 mg; niacin, 22 mg; and menadione sodium bisulfite, 2.33 mg
[4]Provided as milligrams per kilogram of complete diet: Mn, 75 from MnO; Fe, 75 from $FeSO_4 \cdot 7H_2O$; Zn, 75 from ZnO; Cu, 5 from $CuSO_4 \cdot 5H_2O$; I, 0.75 from ethylene diamine dihydroiodide; and Se, 0.1 from Na_2SeO_3.
[5]Provided as % of basal diet: 0.55 L-arginine; 0.24 L-histidine; 0.38 L-isoleucine; 0.67 L-leucine; 0.79 L-lysine HCL; 0.39 L-phenylalanine; 0.22 L-tyrosine; 0.42 L-threonine; 0.07 L-tryptophan; 0.47 L-valine; 0.33 L-serine; 0.32 L-glycine; 0.43 L-proline; 0.79 L-aspartic acid; 1.37 L-glutamic acid; 0.36 L-alanine.
[6]Provided as % of basal diet: 0.61 L-arginine; 0.15 L-histidine; 0.43 L-isoleucine; 0.49 L-leucine; 0.87 L-lysine HCL; 0.35 L-phenylalanine; 0.27 L-tyrosine; 0.43 L-threonine; 0.08 L-tryptophan; 0.51 L-valine; 0.34 L-serine; 0.32 L-glycine; 0.43 L-proline; 0.80 L-aspartic acid; 1.38 L-glutamic acid; 0.36 L-alanine.

Table 2.3. Calculated and analyzed nutrient composition of the experimental grower (d 10 – 24) diets fed to broilers in Experiment 1[1]

Item, % as-fed	LTSAA	Met0	Met1	Met2	Met3	Met4	Met5
Calculated composition							
AME_n, kcal/kg	3171	3165	3165	3165	3165	3165	3166
CP	16.43	16.70	16.74	16.78	16.82	16.87	16.91
Lys	1.16	1.16	1.16	1.16	1.16	1.16	1.16
Met	0.15	0.15	0.22	0.29	0.36	0.43	0.51
Cys	0.16	0.52	0.52	0.52	0.52	0.52	0.52
Met + Cys	0.30	0.66	0.73	0.80	0.88	0.95	1.02
Thr	0.79	0.79	0.79	0.79	0.79	0.79	0.79
Ca	0.93	0.93	0.93	0.93	0.93	0.93	0.93
Non-phytate P	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Analyzed composition							
CP	16.65	17.47	17.07	17.18	17.79	16.96	16.73
Met	0.14	0.16	0.20	0.28	0.34	0.44	0.49
Supp. DL-Met[2]	<0.01	<0.01	0.06	0.12	0.18	0.27	0.34
Cys	0.15	0.50	0.50	0.50	0.49	0.49	0.50
Met + Cys	0.29	0.66	0.71	0.77	0.84	0.93	0.99
Lys	1.04	1.16	1.06	1.09	1.18	1.03	1.01
Thr	0.76	0.76	0.74	0.75	0.78	0.78	0.77

[1]Abbreviations: AME_n, nitrogen-corrected apparent metabolizable energy; supp., supplemental.
[2]Supplemental DL-Met refers to the analyzed level of supplemental DL-Met found in the diet and does not include Met from intact protein sources (i.e., corn and soybean meal).

Table 2.4. Calculated and analyzed nutrient composition of the experimental grower (d 10 – 24) diets fed to broilers in Experiment 2[1]

Item, % as-fed	LTSAA	Met0	Met1	Met2	Met3	Met4	Met5	HMTBA1	HMTBA2	HMTBA3	HMTBA4	HMTBA5
Calculated composition												
AME_n, kcal/kg	3172	3166	3167	3166	3165	3163	3162	3167	3166	3165	3164	3163
CP	16.64	16.90	16.94	16.98	17.02	17.07	17.11	16.90[2]	16.90[2]	16.90[2]	16.90[2]	16.90[2]
Lys	1.23	1.23	1.23	1.23	1.23	1.23	1.23	1.23	1.23	1.23	1.23	1.23
Met	0.15	0.15	0.22	0.29	0.36	0.44	0.51	0.22[3]	0.29[3]	0.36[3]	0.44[3]	0.51[3]
Cys	0.15	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51
Met + Cys	0.30	0.66	0.73	0.81	0.88	0.95	1.02	0.73	0.81	0.88	0.95	1.02
Thr	0.81	0.81	0.81	0.81	0.81	0.81	0.81	0.81	0.81	0.81	0.81	0.81
Ca	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.93
Non-phytate P	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Analyzed composition												
CP	16.35	16.82	17.42	17.01	17.43	17.18	16.74	16.97	17.17	17.12	17.51	17.04
Met	0.14	0.15	0.22	0.29	0.36	0.40	0.50	0.16	0.17	0.16	0.18	0.16
Supp. DL-Met[4]	<0.01	<0.01	0.07	0.12	0.20	0.27	0.35	<0.01	<0.01	<0.01	<0.01	<0.01
Supp. DL-HMTBA[5]	ND	ND	ND	ND	ND	ND	ND	0.10	0.19	0.30	0.39	0.51
Cys	0.17	0.52	0.52	0.51	0.53	0.52	0.53	0.50	0.50	0.53	0.51	0.54
Met + Cys	0.31	0.67	0.74	0.8	0.89	0.93	1.03	0.66	0.67	0.69	0.69	0.70
Lys	1.11	1.11	1.16	1.12	1.1	1.17	1.11	1.16	1.15	1.14	1.19	1.12
Thr	0.74	0.76	0.77	0.77	0.77	0.76	0.76	0.77	0.76	0.79	0.80	0.76

[1]Abbreviations: AME_n, nitrogen-corrected apparent metabolizable energy; supp., supplemental; ND, not detectable.
[2]The amount of crude protein contributed by supplemental DL-HMTBA was not determined, and therefore not accounted for in the calculated value. However, we assume crude protein from supplemental DL-HMTBA to be a small amount (<1% supplemental DL-HMTBA in all diets) and reflect similar values for calculated crude protein from diets Met1 – Met5.
[3]To calculate total Met of the HMTBA treatments, two factors were considered, 1) amount of DL-HMTBA in the silica carrier product (58.52%) and 2) the difference in bioefficacy values of DL-HMTBA relative to DL-Met (70%).
[4]Supplemental DL-Met refers to the analyzed level of supplemental DL-Met found in the diet and does not include Met from intact protein sources (i.e., corn and soybean meal).
[5]Supplemental DL-HMTBA refers to the analyzed level of supplemental DL-HTMBA found in the diet and does not include DL-HMTBA from intact protein sources (i.e., corn and soybean meal).

Table 2.5. Body weight gain, feed intake, and gain:feed of broilers fed graded levels of DL-Met during Experiment 1[1]

	Item	Body weight gain, g/bird			Feed intake, g/bird			Gain:feed, g/kg		
Diet	Phase	0-10	10-24	0-24	0-10	10-24	0-24	0-10	10-24	0-24
LTSAA		206	148[e]	354[e]	242	705[cd]	941[dc]	853	213[d]	398[d]
Met0		193	115[e]	308[e]	237	616[d]	850[d]	841	190[d]	387[d]
Met1		211	325[d]	536[d]	243	765[c]	1008[c]	868	426[c]	533[c]
Met2		204	654[c]	875[c]	242	1118[b]	1359[b]	864	600[b]	642[b]
Met3		206	759[b]	965[b]	238	1186[ab]	1423[ab]	864	640[a]	678[a]
Met4		201	767[b]	967[b]	249	1194[ab]	1433[ab]	841	642[a]	675[a]
Met5		206	814[a]	1020[a]	242	1272[a]	1513[a]	853	641[a]	675[a]
Pooled SEM		5.79	13.82	17.63	6.13	37.87	42.34	15.11	11.19	11.66
Overall model		0.437	<0.001	<0.001	0.989	<0.001	<0.001	0.707	<0.001	<0.001

[a-e]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are least-square means initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment. Average body weight at study initiation was 36 ± 3 g. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 24): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). Abbreviations: SEM, standard error of the mean.

Table 2.6. Estimated requirement of supplemental Met for body weight gain and gain:feed during the grower phase (d 10 – 24) from broilers fed graded levels of DL-Met or DL-HMTBA during Experiment 1 and 2[1]

	Body weight gain, g/bird			Gain:feed, g/kg		
Exp.	Equations	R^2	Req., %	Equations	R^2	Req., %
1	For $x \leq 0.18$, $\hat{y} = 780 - 3867 \times (0.18 - \textit{DL-Met})$ For $x > 0.18$, $\hat{y} = 780$	0.98	0.18	For $x \leq 0.15$, $\hat{y} = 641 - 2857 \times (0.15 - \textit{DL-Met})$ For $x > 0.15$, $\hat{y} = 641$	0.99	0.15
2	For $x \leq 0.19$, $\hat{y} = 801 - 3813 \times (0.19 - \textit{DL-Met})$ For $x > 0.19$, $\hat{y} = 801$	0.97	0.19	For $x \leq 0.12$, $\hat{y} = 605 - 3220 \times (0.12 - \textit{DL-Met})$ For $x > 0.12$, $\hat{y} = 605$	0.96	0.12
2	For $x \leq 0.22$, $\hat{y} = 778 - 3131 \times (0.22 - \textit{DL-HMTBA})$ For $x > 0.22$, $\hat{y} = 778$	0.97	0.22	For $x \leq 0.17$, $\hat{y} = 612 - 2372 \times (0.17 - \textit{DL-HMTBA})$ For $x > 0.17$, $\hat{y} = 612$	0.95	0.17

[1]All diets were supplemented with a fixed L-cystine level of 0.36% and graded levels of supplemental DL-Met or DL-HMTBA. For Experiment 1 and 2, the Met0 diet was calculated to contain 0.15% of total Met and 0.52% of total cysteine and 0.15% of total Met and 0.51% of total cysteine, respectively. A 1-slope broken-line regression model was fitted to the data and used to estimate the requirement of supplemental Met. Individual replicates initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment were used for the regression analysis. Abbreviations: exp., experiment; req., requirement.

Table 2.7. Absolute organ weights from broilers fed graded levels of DL-Met during Experiment 1[1]

	Organ											
Diet	Duod.	Jej.	Ileum	Ceca	Colon	Spleen	Liver	Bursa	Gizz.	Prov.	Crop	Panc.
LTSAA	4.07^{c}	6.42^{c}	5.49^{cd}	3.79^{d}	0.67^{e}	0.57^{c}	16.12^{c}	0.48^{bc}	11.90^{d}	2.73^{d}	1.90^{c}	1.24^{b}
Met0	4.20^{c}	6.76^{c}	3.97^{d}	4.16^{cd}	0.68^{de}	0.36^{c}	15.63^{c}	0.33^{c}	11.25^{d}	2.85^{d}	1.89^{c}	1.50^{b}
Met1	5.67^{b}	10.56^{b}	6.95^{c}	5.79^{bcd}	0.96^{cd}	0.65^{bc}	22.14^{b}	0.79^{bc}	16.22^{c}	3.88^{c}	2.64^{c}	2.47^{a}
Met2	7.28^{a}	18.01^{a}	13.40^{b}	7.94^{ab}	1.24^{c}	0.99^{ab}	30.07^{a}	0.85^{b}	22.63^{b}	5.64^{b}	2.75^{bc}	2.80^{a}
Met3	7.55^{a}	17.10^{a}	12.97^{b}	9.90^{a}	1.46^{ab}	1.26^{a}	32.67^{a}	1.37^{a}	24.39^{ab}	5.80^{ab}	4.28^{a}	2.66^{a}
Met4	7.82^{a}	19.14^{a}	15.27^{a}	8.41^{ab}	1.60^{a}	1.04^{a}	30.82^{a}	1.57^{a}	26.05^{a}	6.08^{ab}	3.55^{ab}	2.47^{a}
Met5	7.32^{a}	18.63^{a}	15.09^{a}	6.78^{bc}	1.50^{ab}	1.11^{a}	32.14^{a}	1.55^{a}	24.50^{ab}	6.69^{a}	4.07^{a}	2.67^{a}
Pooled SEM	0.27	0.87	0.56	1.01	0.12	0.14	1.48	0.26	1.07	0.35	0.34	0.16
Overall model	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a-e]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are measured in grams, were recorded at study conclusion d 24, and represent least-square means initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 24): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). Abbreviations: Duod., duodenum; Jej., jejunum; Gizz., gizzard; Prov., proventriculus; Panc., pancreas; SEM, standard error of the mean.

Table 2.8. Relative weight of organs from broilers fed graded levels of DL-Met during Experiment 1[1]

	Organ											
Diet	Duod.	Jej.	Ileum	Ceca	Colon	Spleen	Liver	Bursa	Gizz.	Prov.	Crop	Panc.
LTSAA	1.10^{ab}	1.57	1.50^{a}	1.03	0.18^{ab}	0.15	4.12^{b}	0.13	3.22^{ab}	0.74	0.52^{ab}	0.38^{b}
Met0	1.19^{a}	2.02	1.19^{c}	1.22	0.21^{a}	0.11	4.71^{a}	0.10	3.39^{a}	0.70	0.58^{a}	0.39^{b}
Met1	1.02^{b}	1.91	1.26^{bc}	1.04	0.17^{abc}	0.12	3.99^{b}	0.14	2.93^{b}	0.70	0.48^{abc}	0.45^{a}
Met2	0.82^{c}	2.01	1.50^{a}	0.90	0.14^{c}	0.11	3.37^{c}	0.10	2.54^{c}	0.63	0.37^{bc}	0.32^{c}
Met3	0.75^{cd}	1.70	1.30^{abc}	0.99	0.15^{bc}	0.12	3.25^{c}	0.14	2.44^{c}	0.58	0.38^{bc}	0.26^{d}
Met4	0.78^{cd}	1.90	1.51^{a}	0.84	0.16^{bc}	0.10	3.06^{c}	0.16	2.43^{c}	0.60	0.35^{c}	0.24^{d}
Met5	0.70^{d}	1.78	1.44^{ab}	0.78	0.16^{bc}	0.12	3.07^{c}	0.19	2.33^{c}	0.64	0.39^{bc}	0.25^{d}
Pooled SEM	0.04	0.12	0.08	0.14	0.02	0.02	0.17	0.03	0.14	0.05	0.06	0.02
Overall model	<0.001	0.067	0.023	0.366	0.025	0.726	<0.001	0.269	<0.001	0.094	0.048	<0.001

[a-d]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are reported as relative weight of an organ as a percentage of body weight, were recorded at study conclusion d 24, and represent least-square means initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 24): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). Relative weight was calculated by taking the weight of the organ (g), dividing it by the live body weight of the bird (g), and then multiplying by 100. Abbreviations: Duod., duodenum; Jej., jejunum; Gizz., gizzard; Prov., proventriculus; Panc., pancreas; SEM, standard error of the mean.

Table 2.9. Absolute and relative length of organs from broilers fed graded levels of DL-Met during Experiment 1[1]

Item	Absolute length, cm						Relative length, %[2]		
Diet Organ	Duodenum	Jejunum	Ileum	Ceca[3]	Colon	Total tract[4]	Duodenum	Jejunum	Ileum
LTSAA	20.5^{d}	40.2^{d}	40.5^{c}	20.2^{cd}	4.5^{c}	95.8^{d}	20.78^{a}	41.38	37.84^{c}
Met0	18.9^{d}	42.9^{cd}	38.3^{c}	18.6^{d}	4.7^{c}	101.1^{d}	19.67^{ab}	42.34	37.98^{c}
Met1	21.0^{cd}	45.3^{c}	45.1^{b}	22.7^{c}	5.6^{b}	111.4^{c}	18.86^{bc}	40.59	40.55^{b}
Met2	23.1^{b}	53.3^{ab}	52.5^{a}	26.2^{b}	6.1^{ab}	128.9^{b}	17.93^{cd}	41.36	40.71^{ab}
Met3	25.2^{a}	56.0^{a}	55.5^{a}	28.9^{a}	6.6^{a}	136.6^{a}	18.42^{bcd}	40.95	40.63^{b}
Met4	22.4^{bc}	50.9^{b}	54.0^{a}	25.5^{b}	5.9^{ab}	127.0^{b}	17.45^{d}	40.02	42.53^{a}
Met5	23.0^{b}	53.5^{ab}	54.8^{a}	26.8^{ab}	6.7^{a}	131.4^{ab}	17.58^{d}	40.74	41.68^{ab}
Pooled SEM	0.57	1.66	1.34	0.87	0.28	3.17	0.48	0.69	0.66
Overall model	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.295	<0.001

[a-d]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are reported as absolute length in centimeters or relative length as a percentage of the total tract length, were recorded at study conclusion d 24, and represent least-square means initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 24): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). Abbreviations: SEM, standard error of the mean.

[2] Relative length was calculated by taking the length of the organ (cm), dividing it by the total tract length (cm), and then multiplying by 100.

[3]The length of the ceca was calculated by taking the length of 1 cecum plus the length of the other cecum.

[4]Total tract length was calculated by adding the absolute lengths of the duodenum, jejunum, and ileum in centimeters.

Table 2.10. Body weight gain, feed intake, and gain:feed of broilers fed graded levels of DL-Met or DL-HMTBA during Experiment 2[1]

Item	Body weight gain, g/bird			Feed intake, g/bird			Gain:feed, g/kg		
Diet Phase	0-10	10-24	0-24	0-10	10-24	0-24	0-10	10-24	0-24
LTSAA	231	165^{f}	400^{e}	265	596^{e}	836^{f}	872	277^{d}	480^{d}
Met0	234	104^{g}	337^{e}	267	480^{f}	738^{f}	877	217^{e}	457^{d}
Met1	244	324^{e}	567^{d}	266	722^{d}	959^{e}	918	449^{c}	592^{c}
Met2	235	656^{d}	890^{c}	264	1099^{c}	1300^{d}	892	597^{ab}	684^{ab}
Met3	244	797^{abc}	1040^{ab}	272	1351^{a}	1542^{a}	898	590^{b}	675^{b}
Met4	249	829^{a}	1078^{a}	273	1335^{a}	1525^{ab}	913	621^{ab}	707^{a}
Met5	240	777^{abc}	1023^{ab}	261	1271^{ab}	1445^{abc}	921	612^{ab}	708^{a}
HMTBA1	239	358^{e}	596^{d}	267	781^{d}	1014^{e}	898	461^{c}	590^{c}
HMTBA2	234	749^{c}	981^{b}	253	1209^{b}	1391^{dc}	902	620^{ab}	705^{ab}
HMTBA3	236	808^{ab}	1042^{ab}	266	1279^{ab}	1470^{abc}	888	631^{a}	709^{a}
HMTBA4	240	774^{abc}	1014^{ab}	261	1297^{ab}	1471^{abc}	904	600^{ab}	691^{ab}
HMTBA5	234	752^{bc}	986^{b}	255	1262^{ab}	1429^{bc}	915	599^{ab}	691^{ab}
Pooled SEM	6.87	21.28	25.77	7.38	38.31	39.46	13.59	13.71	12.02
Overall model	0.622	<0.001	<0.001	0.563	<0.001	<0.001	0.139	<0.001	<0.001

[a-g]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are least-square means initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment. Average body weight at study initiation was 43 ± 5 g. Broilers were fed 1 of 12 low-CP dietary treatments during the grower phase (d 10 – 24): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5) or DL-HMTBA (0.103% increments; HMTBA1 – HMTBA5). Abbreviations: SEM, standard error of the mean.

Table 2.11. Absolute weight of organs from broilers fed graded levels of DL-Met or DL-HMTBA during Experiment 2[1]

	Organ											
Diet	Duod.	Jej.	Ileum	Ceca	Colon	Spleen	Liver	Bursa	Gizz.	Prov.	Crop	Panc.
LTSAA	5.21^{d}	9.43^{fg}	8.15^{e}	3.21^{f}	1.02^{cd}	0.34^{ef}	14.01^{f}	0.70^{cd}	13.98^{cd}	3.51^{e}	2.66	1.76^{e}
Met0	4.42^{d}	8.11^{g}	5.11^{f}	3.13^{f}	0.78^{d}	0.30^{f}	13.72^{f}	0.51^{d}	10.90^{d}	2.67^{f}	2.79	1.38^{e}
Met1	7.06^{c}	11.79^{ef}	8.14^{e}	3.97^{ef}	1.22^{c}	0.55^{de}	19.87^{e}	0.80^{cd}	17.98^{b}	4.05^{de}	3.01	2.30^{d}
Met2	8.26^{abc}	17.35^{c}	13.05^{bcd}	7.22^{ab}	1.59^{ab}	0.91^{bc}	26.53^{c}	1.43^{b}	18.73^{b}	5.17^{ab}	3.55	2.86^{abc}
Met3	8.79^{a}	22.79^{a}	15.33^{ab}	6.94^{ab}	1.82^{a}	0.97^{abc}	31.90^{ab}	1.51^{b}	23.81^{a}	5.39^{ab}	4.02	2.43^{cd}
Met4	7.45^{bc}	18.74^{bc}	14.63^{abc}	5.54^{bcde}	1.83^{a}	1.15^{ab}	33.01^{a}	1.66^{ab}	24.64^{a}	5.62^{a}	4.12	2.56^{bcd}
Met5	8.71^{a}	16.30^{cd}	11.08^{d}	6.34^{abc}	1.68^{a}	1.05^{abc}	25.41^{cd}	1.44^{b}	23.83^{a}	5.02^{abc}	3.17	2.75^{abc}
HMTBA1	7.60^{abc}	13.29^{de}	8.31^{e}	4.59^{def}	1.30^{bc}	0.65^{d}	21.66^{de}	0.95^{c}	16.68^{bc}	4.32^{cd}	2.82	2.25^{d}
HMTBA2	8.09^{abc}	21.03^{ab}	15.49^{a}	7.63^{a}	1.75^{a}	1.20^{a}	32.40^{ab}	1.62^{ab}	25.64^{a}	5.21^{ab}	4.21	2.90^{ab}
HMTBA3	8.66^{ab}	16.94^{c}	12.79^{cd}	5.21^{cde}	1.71^{a}	1.04^{abc}	29.21^{abc}	1.75^{ab}	25.01^{a}	5.49^{ab}	3.04	2.56^{bcd}
HMTBA4	8.58^{ab}	19.37^{abc}	16.06^{a}	6.15^{abcd}	1.82^{a}	0.96^{bc}	28.50^{bc}	1.96^{a}	22.93^{a}	5.26^{ab}	3.77	3.08^{a}
HMTBA5	8.21^{abc}	17.76^{bc}	12.90^{cd}	6.05^{abcd}	1.67^{a}	0.91^{c}	28.29^{bc}	1.38^{b}	22.84^{a}	4.90^{bc}	3.11	2.53^{bcd}
Pooled SEM	0.46	1.33	0.89	0.65	0.13	0.09	1.60	0.16	1.50	0.27	0.44	0.16
Overall model	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.050	< 0.001

[a-g]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are measured in grams, were recorded at study conclusion d 24, and represent least-square means initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 12 low-CP dietary treatments during the grower phase (d 10 – 24): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5) or DL-HMTBA (0.103% increments; HMTBA1 – HMTBA5). Abbreviations: Duod., duodenum; Jej., jejunum; Gizz., gizzard; Prov., proventriculus; Panc., pancreas; SEM, standard error of the mean.

Table 2.12. Relative weight of organs from broilers fed graded levels of DL-Met or DL-HMTBA during Experiment 2[1]

	Organ											
Diet	Duod.	Jej.	Ileum	Ceca	Colon	Spleen	Liver	Bursa	Gizz.	Prov.	Crop	Panc.
LTSAA	1.17^{a}	2.09^{ab}	1.83^{a}	0.72^{ab}	0.21^{ab}	0.08	3.34^{ab}	0.16	3.15^{a}	0.81^{a}	0.59^{ab}	0.39^{a}
Met0	1.19^{a}	2.17^{a}	1.37^{bc}	0.84^{a}	0.19^{abcd}	0.08	3.66^{a}	0.13	2.93^{ab}	0.71^{ab}	0.63^{a}	0.37^{ab}
Met1	1.15^{a}	1.93^{abc}	1.33^{bc}	0.65^{abc}	0.20^{abc}	0.09	3.23^{bc}	0.13	2.92^{ab}	0.66^{bc}	0.49^{abc}	0.38^{ab}
Met2	0.91^{b}	1.90^{abcd}	1.41^{b}	0.79^{ab}	0.18^{abcd}	0.10	2.89^{cde}	0.16	2.09^{d}	0.57^{cd}	0.39^{cde}	0.31^{cd}
Met3	0.81^{bc}	2.09^{ab}	1.41^{b}	0.65^{abc}	0.17^{bcd}	0.09	2.90^{cde}	0.14	2.19^{cd}	0.50^{de}	0.37^{cde}	0.22^{g}
Met4	0.71^{c}	1.65^{cde}	1.28^{bc}	0.49^{c}	0.16^{d}	0.10	2.91^{cde}	0.17	2.18^{cd}	0.50^{de}	0.37^{cde}	0.23^{g}
Met5	0.81^{bc}	1.51^{e}	1.03^{d}	0.59^{bc}	0.16^{d}	0.10	2.37^{f}	0.13	2.23^{cd}	0.47^{de}	0.30^{de}	0.26^{efg}
HMTBA1	1.14^{a}	1.94^{abc}	1.31^{bc}	0.75^{ab}	0.21^{a}	0.11	3.29^{b}	0.15	2.64^{bc}	0.65^{bc}	0.46^{bcd}	0.34^{bc}
HMTBA2	0.76^{c}	1.98^{abc}	1.46^{b}	0.72^{ab}	0.17^{cd}	0.11	3.04^{bcd}	0.15	2.42^{cd}	0.49^{de}	0.40^{cde}	0.27^{def}
HMTBA3	0.79^{bc}	1.56^{de}	1.17^{cd}	0.48^{c}	0.16^{d}	0.10	2.69^{ef}	0.16	2.30^{cd}	0.51^{de}	0.28^{e}	0.24^{fg}
HMTBA4	0.79^{bc}	1.79^{bcde}	1.48^{b}	0.58^{bc}	0.17^{bcd}	0.09	2.61^{ef}	0.18	2.12^{d}	0.43^{e}	0.47^{abcd}	0.29^{de}
HMTBA5	0.80^{bc}	1.75^{bcde}	1.26^{bc}	0.60^{bc}	0.16^{cd}	0.09	2.77^{de}	0.13	2.23^{cd}	0.48^{de}	0.31^{de}	0.25^{efg}
Pooled SEM	0.05	0.14	0.08	0.08	0.01	0.01	0.13	0.02	0.17	0.04	0.06	0.02
Overall model	< 0.001	0.003	< 0.001	0.016	0.043	0.080	< 0.001	0.641	< 0.001	< 0.001	0.001	< 0.001

[a-g]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are reported as relative weight of an organ as a percentage of body weight, were recorded at study conclusion d 24 and represent least-square means initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 12 low-CP dietary treatments during the grower phase (d 10 – 24): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5) or DL-HMTBA (0.103% increments; HMTBA1 – HMTBA5). Relative weight was calculated by taking the weight of the organ (g), dividing it by the live body weight of the bird (g), and then multiplying by 100. Abbreviations: Duod., duodenum; Jej., jejunum; Gizz., gizzard; Prov., proventriculus; Panc., pancreas; SEM, standard error of the mean.

Table 2.13. Absolute and relative length of organs from broilers fed graded levels of DL-Met or DL-HMTBA during Experiment 2[1]

Item	Absolute length, cm						Relative length, %[2]		
Diet Organ	Duodenum	Jejunum	Ileum	Ceca[3]	Colon	Total tract[4]	Duodenum	Jejunum	Ileum
LTSAA	20.4^{cd}	42.3^{e}	41.6^{de}	20.3^{e}	4.7^{b}	104.3^{f}	19.67	40.57	39.76
Met0	20.1^{d}	42.9^{de}	39.0^{e}	17.6^{f}	4.0^{c}	102.0^{f}	19.75	42.05	38.21
Met1	23.9^{ab}	47.0^{de}	44.6^{cd}	21.4^{de}	5.3^{ab}	115.5^{e}	20.70	40.71	38.59
Met2	24.1^{ab}	49.0^{bcd}	48.2^{abc}	24.8^{bc}	4.6^{bc}	121.4^{cde}	19.86	40.44	39.65
Met3	23.9^{ab}	56.2^{a}	51.4^{a}	26.9^{ab}	5.1^{ab}	131.4^{ab}	18.27	42.57	39.16
Met4	22.9^{bc}	53.4^{abc}	51.7^{a}	24.6^{c}	5.1^{ab}	127.9^{abcd}	17.97	41.59	40.45
Met5	25.7^{a}	54.3^{ab}	49.7^{ab}	24.6^{c}	5.4^{a}	129.6^{abc}	19.83	41.79	38.38
HMTBA1	23.2^{ab}	46.2^{de}	45.4^{bcd}	20.7^{e}	5.1^{ab}	114.8^{e}	20.41	40.16	39.43
HMTBA2	23.6^{ab}	56.6^{a}	52.5^{a}	27.6^{a}	5.2^{ab}	131.4^{ab}	18.20	43.03	39.93
HMTBA3	25.1^{ab}	48.7^{bcd}	48.8^{abc}	23.7^{c}	5.4^{a}	122.5^{bcde}	20.47	39.67	39.86
HMTBA4	23.7^{ab}	56.2^{a}	53.3^{a}	24.7^{c}	5.3^{a}	133.2^{a}	17.85	42.19	39.96
HMTBA5	23.1^{abc}	47.4^{cde}	48.6^{abc}	22.8^{cd}	5.4^{a}	119.1^{de}	19.37	41.83	39.45
Pooled SEM	1.05	2.44	1.99	0.83	0.24	3.73	0.90	1.09	0.96
Overall model	0.005	< 0.001	< 0.001	< 0.001	0.001	<0.001	0.161	0.395	0.810

[a-f]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are reported as absolute length in centimeters or relative length of an organ as a percentage of the total tract length, were recorded at study conclusion d 24, and represent least-square means initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 12 low-CP dietary treatments during the grower phase (d 10 – 24): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cystine (Met0), the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5) or DL-HMTBA (0.103% increments; HMTBA1 – HMTBA5). Abbreviations: SEM, standard error of the mean.

[2]Relative length was calculated by taking the length of the organ (cm), dividing it by the total tract length (cm), and then multiplying by 100.

[3]The length of the ceca was calculated by taking the length of 1 cecum plus the length of the other cecum in centimeters.

[4]Total tract length was calculated by adding the duodenum, jejunum, and ileum in centimeters.

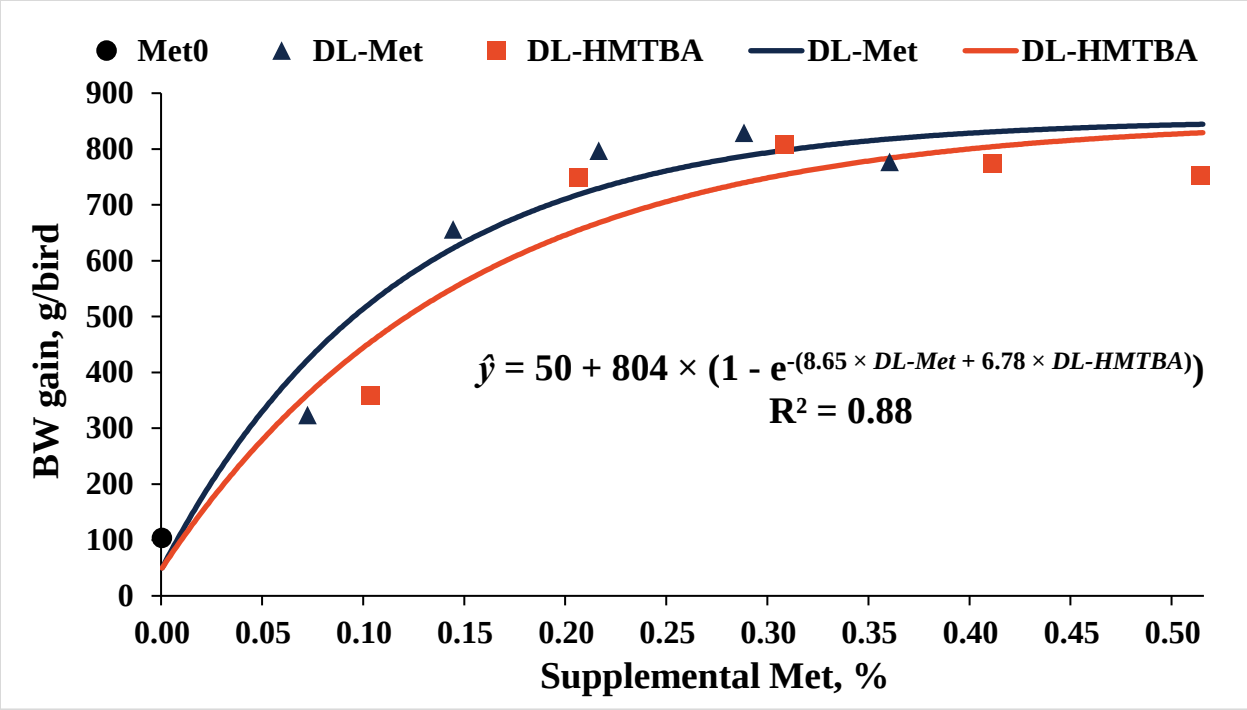

Figure 2.1. Bioavailability of supplemental DL-HMTBA relative to DL-Met (% of diet) for the body weight (BW) gain (g/bird) of broilers fed graded levels of DL-Met or DL-HMTBA during the grower phase (d 10 – 24) of Experiment 2. The pooled SEM was 35.3. Values are least-square means initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment. The LTSAA birds were removed from the data set to model a dose-response. A nonlinear exponential regression model was fitted to the data and used to estimate the bioavailability of supplemental DL-HMTBA relative to DL-Met for BW gain. The bioavailability of DL-HMTBA was estimated to be 78.4%.

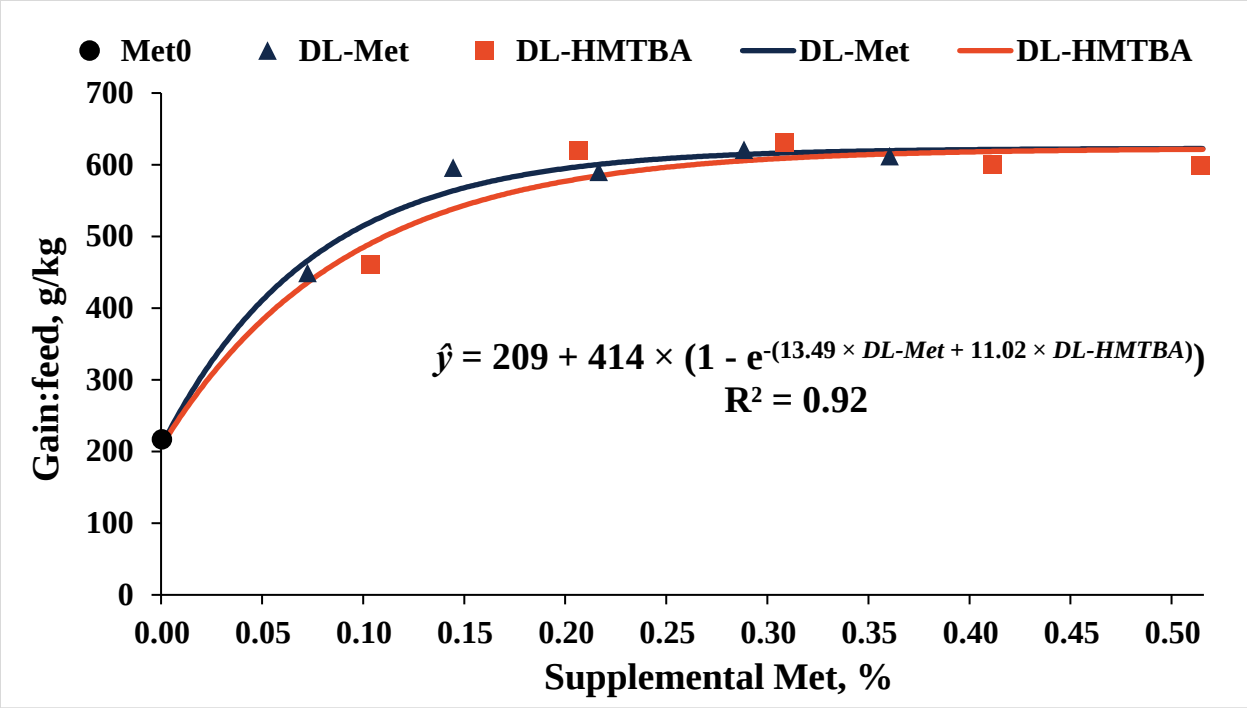

Figure 2.2. Bioavailability of supplemental DL-HMTBA relative to DL-Met (% of diet) for the gain:feed (g/kg) of broilers fed graded levels of DL-Met or DL-HMTBA during the grower phase (d 10 – 24) of Experiment 2. The pooled SEM was 15.8. Values are least-square means initially derived from 6 birds per replicate cage, 7 replicate cages per dietary treatment. The LTSAA birds were removed from the data set to model a dose-response. A nonlinear exponential regression model was fitted to the data and used to estimate the bioavailability of supplemental DL-HMTBA relative to DL-Met for gain:feed. The bioavailability of DL-HMTBA was estimated to be 81.7%.

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CHAPTER 3: EFFECTS OF DIETARY METHIONINE DURING AN ACUTE INFLAMMATORY CHALLENGE ON BROILER GROWTH AND IMMUNE PERFORMANCE AND THE REQUIREMENT OF METHIONINE

Abstract: An experiment was conducted to examine the impact of dietary Met on the growth and immune performance of broilers and the requirement of supplemental DL-Met during an acute inflammatory challenge. A total of 490 Ross 308 male broilers were allotted to 7 treatments with 7 replicates cages per treatment, 10 chicks per replicate cage, and fed a standard broiler starter diet from d 0 – 10. At d 10, broilers were switched to 1 of 7 low-CP experimental grower (d 10 – 28) dietary treatments that included a low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). At study d 25, all birds were challenged *in vivo* intraperitoneally (i.p.) with lipopolysaccharide (LPS) derived from *E. coli* at 2.0 mg/kg of BW. At -24, 5, 24, and 72 hours post-stimulation (HPS) with LPS, 1 bird per pen was euthanized to permit the collection of body temperature, blood for hematology, absolute and relative liver DM weights, and, with the exclusion of 72 HPS, liver and spleen for quantifying the relative gene expression of the cytokines interleukin-1 beta (*IL-1β*), interleukin-6 (*IL-6*), and interleukin-10 (*IL-10*). Additionally, at 24 HPS, the spleen was collected for isolation of splenic macrophages used in an *ex vivo* stimulation assay where IL-6 protein concentration was quantified. Growth performance was improved ($P < 0.001$) with the addition of supplemental DL-Met before (d 10 – 24), during (d 24 – 26), and after (d 26 – 28) the *in vivo* LPS challenge. The estimated requirement of supplemental DL-Met from the 1-slope broken-line regression analysis numerically decreased for BW gain and gain:feed during the *in vivo* LPS challenge (d 24 – 26) by about 7% and 39%, respectively, compared to the requirement of supplemental DL-

Met during the pre-challenge period (d 10 – 24). The ratio of heterophils to lymphocytes (H:L) decreased when at least Met3 or above was fed to broilers at all time-points following the *in vivo* LPS challenge. Hepatic and splenic mRNA expression of *IL-6*, *IL-1β*, and *IL-10* was independently impacted ($P < 0.001$) by HPS. Hepatic *IL-6* and *IL-1β*, and splenic *IL-1β* were differentially impacted ($P < 0.016$) by dietary treatment or the level of supplemental DL-Met dependent on HPS. Hepatic *IL-6* mRNA expression was increased ($P < 0.008$) at 5 HPS with an FC of over 197 and 172 for Met2 and Met4, respectively, compared to all other dietary treatments and time-points. Dietary treatment had no impact ($P > 0.05$) on broiler body temperature, although the main effect of HPS significantly impacted ($P < 0.001$) body temperature, indicating an activated immune system. Absolute and relative liver DM weights were significantly impacted ($P < 0.010$) by dietary treatment at -24, 5, and 72 HPS. At 24 HPS, absolute liver DM weight was impacted ($P < 0.001$) by dietary treatment, while relative liver DM was not. Dietary treatment had no impact ($P > 0.05$) on isolated splenic macrophage IL-6 production. Taken together, these findings suggest that Met ingestion during the onset of an innate response may help initiate a robust acute phase response within the liver. Additionally, more supplemental DL-Met may be required to reduce stress as measured by the H:L ratio than what is required for optimal growth. Further research should be directed towards investigating the impact of dietary Met on innate immunity of broilers and whether improvements made to immunity can influence broiler health long-term.

Introduction

An immune response is required for overcoming immune challenges, but activating, utilizing, and maintaining a robust immune system does not come without metabolic cost. As a broiler's immune system becomes activated, nutrient partitioning is altered to prioritize

maintenance and immune function (Humphrey and Klasing, 2004). During this period, feed intake and growth performance can be reduced (Cao et al., 2013; Rochell et al., 2016; Park et al., 2020). The immune system utilizes amino acids directly and as substrates for proteins, immune cells, nucleic acids, polyamines, neurotransmitters, and anti-oxidants (Li et al., 2007). Currently, amino acid recommendations are typically estimated from broilers reared in non-challenged conditions using growth parameters as efficiency measures. This method fails to consider changes in amino acid metabolism upon immune activation. Therefore, determining the requirement of amino acids during immune activation may mitigate immune-related reductions in growth performance and aid in restoring homeostasis.

The importance of the amino acid Met is well known in broiler nutrition primarily for its limiting nature and routine supplementation to the diet (Fernandez et al., 1994). However, lesser-known are its roles in immune function. Like other amino acids, Met is utilized in protein synthesis, but unique to Met is its inclusion in the production of formylmethionine, the initiator of protein synthesis (D'Mello, 2003). Generation of diverse proteins (e.g., cytokines, acute phase proteins, complement proteins) and immune cells (e.g., granulocytes, immunoglobulins, macrophages, natural killer cells, lymphocytes) also requires Met. The metabolite *S*-adenosylmethionine is formed from Met and is the primary methyl donor for biological reactions. Methylation of cell phospholipids helps maintain membrane fluidity and methylation of DNA regulates gene transcriptional activity of immune functions like production of the pro-inflammatory cytokines interleukin 1-β (*IL-1β*), interferon-gamma, and interleukin-17A, mediation of natural killer cells, complement activation, and proliferation, differentiation, and regulation of T and B cells (Lu, 2000; Suarez-Alvarez et al., 2012). Additionally, Met is important in synthesizing polyamines, an essential compound for cell growth that can interact

with Toll-like receptors, components of innate immunity (Igarashi and Kashiwagi, 2000; Handa et al., 2018; Hesterberg et al., 2018). The amino acid cysteine is generated from Met metabolism in an irreversible reaction, and both Met and cysteine are required for protein synthesis (i.e., total sulfur amino acid requirement) (Baker and Dilger, 2009). The potent anti-oxidants Tau and glutathione, which help maintain the oxidation status of a bird, are made from cysteine (Wu, 2009). The transcription factor nuclear factor kappa B, a significant regulator of innate immunity, can be influenced by cysteine and Tau (Tesseraud et al., 2009; Surai et al., 2020).

Lipopolysaccharide (LPS) is an endotoxin found in the outer membrane of Gram-negative bacteria and is composed of an O-polysaccharide chain, a core region, and a lipid A structure (Erridge et al., 2002). As a potent activator of innate immunity, sensing of LPS by innate immune cells initiates an acute orchestration of events for overcoming infection. One element is the rapid production of cytokines (Rathinam et al., 2019). Among some of the cytokines produced from LPS-induced immune activation are the pro-inflammatory cytokines interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-alpha, which can signal to the anterior hypothalamus to induce the febrile response and to the liver to initiate the acute phase response (Heinrich et al., 1990; Zampronio et al., 2015). Changes in cytokine production because of immune stimulation also cause unwanted behavior in production animals like lethargy and anorexia (Kelley et al., 2003).

Macrophages are one of the primary innate immune cells that can detect LPS. The macrophage is most notable for its phagocytic features, but it is also essential for producing cytokines, chemokines, and reactive oxygen species, processing and presenting antigens, and possessing anti-microbial and anti-tumoral properties (Dietert and Golemboski, 1998; Qureshi et al., 2000; Qureshi, 2003). Polyamines can directly impact macrophages by influencing their

polarization or the method by which they are activated and function (Murray, 2017; Latour et al., 2020). The spleen is a secondary immune organ where macrophages phagocytize antigens from the blood and where macrophages expose antigens to lymphocytes (Olah et al., 2012). Little is known about the impact of dietary Met levels on the *ex vivo* stimulation response of primary broiler splenic macrophages. However, characterizing the relationship between dietary Met and broiler splenic macrophage activity may elucidate a mechanistic pathway by which dietary Met impacts the innate immune response.

Broilers can reach market weight in as little as 6 weeks, and even marginal disruptions in feed efficiency can significantly impact production that may not be recovered by the time a broiler goes to market. Additionally, a robust innate immune response has been shown to improve a bird's resistance to other pathological diseases (Swaggerty et al., 2011). The immune-related roles of Met suggest the broiler immune response may be sensitive to the level of dietary Met, but the influence of Met on growth and immunity during and after an acute inflammatory challenge is poorly understood. Therefore, the objective of this experiment was to investigate if dietary treatments of graded levels of supplemental DL-Met during an *in vivo* acute immune challenge by LPS impact broiler immune responsivity, *ex vivo* splenic macrophage activity, and the requirement of supplemental DL-Met. We hypothesize that diets supplemented with DL-Met will influence the immune response and promote improved growth performance of broilers during an *in vivo* acute inflammatory challenge to LPS.

Materials and Methods

Before initiating the experiment, all animal husbandry and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois Urbana-Champaign.

Bird Husbandry and Dietary Treatments

A total of 490 day-old Ross 308 male broiler chicks were obtained from a commercial hatchery (Hoovers Hatchery, Rudd, IA) for utilization in an experiment with 7 treatments, 7 replicates per treatment, and 10 chicks per replicate (**Figure 3.1**). Chicks were transported to the University of Illinois before being placed in thermostatically controlled battery cages measuring 99 cm long, 66 cm wide, and 33 cm high (model SB5T; Alternative Design Manufacturing, Siloam Springs, AR) with raised-wire flooring. The rooms housing the battery cages were environmentally controlled according to breeder standards and included continuous lighting. Upon arrival, chicks were placed in battery cages with access to water overnight. Following an overnight fast, at study d 0, chicks were individually weighed (43 ± 4 g), wing-banded, allotted according to initial BW, and randomly assigned to individual treatment groups. All diets were fed in the mash form and provided *ad libitum* in a trough feeder (61 cm long, 9 cm wide, and 9 cm deep), in addition to tap water via 2 industry-standard nipple drinkers. From study d 0 to 10, all chicks were fed a practical corn-soybean meal-based starter diet (**Table 3.1**). On study d 10, all birds were switched to their respective experimental grower diets (**Table 3.2**). The experimental grower diets were fed during the grower phase (d 10 – 28) and included a low total sulfur amino acid diet (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and Met0 with supplemented graded levels of DL-Met (0.072% increments; Met1 – Met5). The supplemental levels were chosen such that the LTSAA and Met0 diets were formulated to be devoid of supplemental DL-Met and contain 29% of the total Met requirement compared to the Ross Broiler: Nutrient Specifications (2019) for as-hatched broilers grown to 3.60 – 4.00 kg of BW for the grower feeding phase (d 11 – 24) while Met5, the diet containing the highest level of supplemental DL-Met, was formulated to contain 100% of the total Met requirement (i.e., the

supplemental levels validated in Chapter 3) (Aviagen, 2019). Compared to the Poultry NRC (1994) for broilers aged 0 – 3 wk, LTSAA and Met0 were formulated to 29% of the total Met requirement, while Met5 was formulated to 101% of the total Met requirement (NRC, 1994). Because the level of dietary cysteine can change the requirement of Met due to its sparing capacity on Met, dietary cysteine was formulated to be fed in slight excess and at an equal level in all dietary treatments used for requirement estimations (i.e., all but LTSAA) (Baker and Dilger, 2009). Supplemental DL-Met (MetAMINO 99%, Evonik Nutrition and Care GmbH, Hanau, Germany) and L-cysteine were added to the diet at the expense of dextrose, while the remainder of ingredients were kept constant across treatments. In the experimental grower diets, the amino acids Arg, Thr, Trp, and Gly, were formulated to be included at approximately 15% over the requirement recommended by AMINOChick 2.0 [Arg, Thr, Trp (Evonik Nutrition and Care GmbH, Hanau, Germany)] or AMINOChick 3.0 [Gly (Evonik Nutrition and Care GmbH, Hanau, Germany)]. This was done to ensure no other amino acids in the diet would be limiting except for Met. The remainder of amino acids were fed adequately and formulated using requirements derived from AMINOChick 2.0 (Evonik Nutrition and Care GmbH, Hanau, Germany). Experimental grower diets were analyzed by AMINO-Lab (Evonik Product Quality & Regulatory Affairs, Hanau, Germany) for crude protein (Association of Official Analytical Chemists (AOAC) method 990.03; AOAC International, 2000) and amino acids (AOAC method 994.12; AOAC International, 2000) using an amino acid analyzer (Biochrom30+; Cambridge, UK) (**Table 3.3**). Analyzed composition values were similar to the calculated composition values. Individual chick weights and pen feeder weights were captured on study d 0, 10, 24, 26, and 28 to calculate BW gain, feed intake, and gain:feed. The BW gain and gain:feed calculated for the grower phase were utilized to estimate supplemental DL-Met requirements. The

requirement for supplemental DL-Met (% of diet) for BW gain and gain:feed were estimated for the following periods: d 10 – 24, 0 – 28, 24 – 26, 26 – 28, and 24 – 28.

In Vivo Immune Stimulation and Sample Collection

On study d 25, all birds were challenged intraperitoneally (i.p.) with lipopolysaccharide (LPS) derived from *E. coli* strain O127:B8 (L3129, Source, 12170803, Batch, 0000082455; Sigma-Aldrich, St. Louis, MO) at 2.0 mg/kg of BW of the bird captured on d 24 (i.e., *in vivo* LPS stimulation). On study d 24 [-24 hours post-stimulation (HPS)], 25 (5 HPS), 26 (24 HPS), and 28 (72 HPS), 1 bird per pen (randomly selected) was chosen to record body temperature via the cloaca using a commercial-grade livestock probe thermometer (GLA M700 Thermometer; GLA Agricultural Electronics, San Luis Obispo, CA). The same bird randomly selected for body temperature was euthanized via CO_2 asphyxiation to permit the collection of whole blood and tissues. Immediately upon euthanasia, birds were bled via cardiac puncture into evacuated 2-mL tubes coated in sodium heparin (454302; Greiner Bio-One GmbH, Kremsmünster, Austria) and kept on ice until submission to the Veterinary Clinical Pathology Laboratory at the University of Illinois Urbana-Champaign for measuring hematocrit, total protein, and as a percentage of white blood cells, band heterophils, heterophils, lymphocytes, monocyte/azurophil granules, eosinophils, and basophils. The ratio of heterophils to lymphocytes was calculated by taking the reported heterophils as a percentage of white blood cells and dividing it by the lymphocytes as a percentage of white blood cells. Following blood collection, the whole liver was removed and weighed for determining absolute liver weight on a DM basis. A sub-sample of the liver was taken after the whole liver weight was captured for determining the percent DM (AOAC method 934.01; AOAC International, 2000). The calculated percent DM of an individual liver was multiplied by the respective raw liver weight to put the absolute liver weight on a DM basis. The

relative weight of the liver was then calculated by taking the weight of the liver on a DM basis (g), dividing it by the live BW of the bird (g), and then multiplying by 100 to represent a percentage of BW.

On study d 14 and 21, all birds were inoculated intramuscularly in the pectoralis major with 0.2 mg of BSA (A3912; Sigma-Aldrich, St. Louis, MO) dissolved in 200 µL of 1x phosphate-buffered saline (2810305; MP Biomedicals, Solon, OH) and either 200 µL of Complete Freund's Adjuvant (d 14) (F5881; Sigma-Aldrich, St. Louis, MO) or Incomplete Freund's Adjuvant (d 21) (F5506; Sigma-Aldrich, St. Louis, MO). On study d 21 and 28 (same collection bird as detailed above), 1 randomly selected bird per pen was euthanized via CO_2 asphyxiation to permit whole blood collection via cardiac puncture for isolation of serum designated for use in an anti-BSA IgY assay. However, upon processing, a significant portion of serum samples (>75%) were gelatinized, and it was determined that there was not enough viable serum to perform the anti-BSA IgY assays. Nevertheless, the BSA assay was applied to all birds at both inoculations time-points to equalize effects across all treatment groups and are not expected to impact any of the other results.

Inflammatory Cytokine mRNA Expression

On study d 24 (-24 HPS), 25 (5 HPS), and 26 (24 HPS), a sub-sample of the liver (after recording the weight) and spleen were collected from 1 randomly selected bird per pen (same collection bird as detailed above) euthanized via CO_2 asphyxiation for quantifying the relative gene expression of *IL-1β*, *IL-6*, and interleukin-10 (*IL-10*) by quantitative real-time polymerase chain reaction (PCR). Immediately following collection, sub-samples were submerged into tubes containing RNA*later* Stabilization Solution (Thermo Fisher Scientific, Waltham, MA) and then placed at 4°C overnight to allow its diffusion into tissues. Tubes containing the samples +

RNA*later* were then moved to –80°C pending further analysis. Samples were analyzed following a similar protocol as previously described (Rochell et al., 2016; Oelschlager et al., 2019; Rasheed et al., 2020). The TaqMan Gene Expression Assay (Thermo Fisher Scientific, Waltham, MA) was used to perform quantitative real-time PCR to quantify relative gene expression of *IL-1β* (NM_204524.1; Thermo Fisher Scientific, Waltham, MA), *IL-6* (NM_204628.1; Thermo Fisher Scientific, Waltham, MA), and *IL-10* (NM_001004414.2; Thermo Fisher Scientific, Waltham, MA). Amplification was achieved by PCR for both target (*IL-1β*, *IL-6*, and *IL-10*) and the reference chicken gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (NM_204305.1; Thermo Fisher Scientific, Waltham, MA) (Hong et al., 2006). Sample cDNA was amplified using TaqMan (4304437; Thermo Fisher Scientific, Waltham, MA) oligonucleotide probes containing 5' fluorescent reporter dye (6-FAM) and 3' non-fluorescent quencher dye, and fluorescence was determined using a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative gene expression was calculated using the comparative threshold cycle (Ct) method (Livak and Schmittgen, 2001). The FC results were first calculated relative to 0 HPS within an individual dietary treatment of a specific gene-tissue combination and then additionally baseline-corrected within a specific gene-tissue combination to dietary treatment Met0 at -24 HPS.

Ex Vivo Cell Stimulation and IL-6 Concentration

On study d 26, a sub-sample of spleen was collected from 1 randomly selected bird per pen (same collection bird as detailed above) euthanized via CO_2 asphyxiation to isolate splenic macrophages using cell gradient centrifugation methods (Histopaque-1077; Sigma-Aldrich, St. Louis, MO). Immediately upon removal of the spleen, the sub-sample (approximately half of the spleen mass) was trimmed and placed directly into a pre-filled plastic collection tube containing

RPMI-1640 medium (R7388; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (F4135; Sigma-Aldrich, St. Louis, MO), 1% 100x MEM non-essential amino acids (M7145; Sigma-Aldrich, St. Louis, MO), and 1% PSN (100x) Antibiotic Mixture containing 5 mg/mL of penicillin, 5 mg/mL of streptomycin, and 10 mg/mL neomycin (15640-055; Gibco, Life Technologies, Inc., Grand Island, NY), and then stored on ice until all tissue collections were complete. Subsequently, spleen sub-samples were poured into a 70-µm cell strainer (CLS431751; Sigma-Aldrich, St. Louis, MO) over a plastic petri dish (AS4052; Thermo Fisher Scientific, Waltham, MA) and crushed through the screen using the plunger from a 3-mL syringe (14-823-40; Thermo Fisher Scientific, Waltham, MA) and supplemented RPMI-1640 medium. The cell solution in the petri dish was then carefully layered over the Histopaque-1077 (10771; Sigma-Aldrich, St. Louis, MO) density gradient in a 1:1 ratio of Histopaque-1077 to cell solution and then centrifuged at 1,800 rpm for at least 20 min at room temperature. The intermediate buffer layer (i.e., mononuclear cells) was collected upon centrifugation and washed twice with supplemented RPMI-1640 medium. Cells were then counted using a MOXI Z Mini Automated Cell Counter (MXZ000; ORFLO Technologies, Bothell, WA) and seeded at 2.0 x 106 cells/ml of supplemented RPMI-1640 medium in 24-well cell culture plates (10062-896; VWR® International, LLC, Radnor, PA) and left to adhere overnight (5% CO2; 41° C). Each spleen sub-sample (i.e., one per bird per cage) was plated twice, one as a control well and one as an LPS treatment well. After overnight adhesion, all cell supernatants were discarded via aspiration and then stimulated with 1 mL of either supplemented RPMI-1640 medium (i.e., control) or LPS (i.e., LPS treatment) derived from Escherichia coli O127:B8 (L3129; Sigma-Aldrich, St. Louis, MO) at 100 µg/mL of supplemented RPMI-1640 and left to incubate (5% CO2; 41° C) for 6 h. Six h post *ex vivo* cell stimulation, cell supernatants were removed and stored at -80° C pending

later analysis. Cell supernatants were analyzed for the concentration of IL-6 by an ELISA kit (ECH6RB; Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. The limit of detection of the IL-6 ELISA kit was 0.085 ng/mL.

Statistical analysis

Growth performance, body temperature, hematology, liver weights, and IL-6 concentration. The experimental design was a completely randomized design. All analyses were a 1-way ANOVA using the MIXED procedure of SAS (version 9.4; SAS Institute, Cary, NC) to determine whether the model was significant. When appropriate, means separation was conducted. Individual cage served as the experimental unit, and dietary treatment was considered the main effect of the model except for body temperature, where both the main effect of treatment and the main effect of HPS were considered. The replicate cage was included as a random effect. In all instances, outliers were identified as having an absolute Studentized residual value of 3 or greater, and significance was accepted at a *P* – value of less than 0.05. The requirement of supplemental DL-Met was estimated using a 1-slope broken-line regression model (Robbins et al., 1979, 2006). The LTSAA group was excluded from all analyses used to estimate the requirement or supplemental DL-Met.

Gene expression. The mRNA expression data were analyzed separately for each gene and tissue based on the following overall linear mixed model (eq. [1]):

$$\Delta CT_{(i)jklm} = \mu + C_i + Met(C)_{(i)j} + H_k + (C * H)_{ik} + [Met(C) * H]_{(i)jk} + B_l + p_m + e_{(i)jklm}$$

Eq. [1]

where ΔCT represents the $\log_2$ relative expression levels of the experimental unit (i.e., cage); μ is the general mean; C_i is the fixed effect of the i^{th} L-cysteine treatment (i = 1 to 2); $Met(C)_{(i)j}$ is the fixed effect of the j^{th} level of supplemental DL-Met [j = 1 (0% of the diet) to 6 (0.36% of the diet)] within the L-cysteine supplemented dietary treatments (i.e., i = 2); H_k is the fixed effect of the k^{th} HPS (k = 1 to 3); $(C * H)_{ik}$ and $[Met(C) * H]_{(i)jk}$ represent interactions between previously defined effects; B_l is the fixed effect of the l^{th} block; p_m is the random effect of the m^{th} cage, assuming $p \sim N(0, \sigma_p^2)$, where σ_p^2 represents the cage variance; and $e_{(i)jklm}$ is the random residual of the m^{th} cage associated with $\Delta CT_{(i)jklm}$, assuming $e \sim N(0, \boldsymbol{I} \otimes \boldsymbol{R})$ where $\boldsymbol{I}$ represents an identity matrix of order equal to the number of cages and $\boldsymbol{R}$ the (co)variance structure of the residuals of order 3 due to repeated records across HPS. Although the first time-point is represented by 24h prior to stimulation (i.e., -24 HPS), statistical analyses assumed this collection at time 0.

The overall mixed model in eq. [1] was evaluated for each combination of gene and tissue to identify the best model and assumptions for each combination. For this, we evaluated models assuming (i) heterogeneous residual variances at each HPS (i.e., $\boldsymbol{R} = \boldsymbol{I}_3 \sigma_{e_k}^2$) and (ii) using traditional (co)variance structures. For (ii), the random effect of cage was removed from the model as the traditional structures evaluated already accounted for the correlations among observations from the same cage. The structures evaluated included: compound symmetry (CS), heterogeneous CS, first-order autoregressive [AR(1)], heterogeneous AR(1), spatial power, and unstructured. Within each gene-tissue combination, the model showing the lowest Akaike Information Criterion (AIC) was used for subsequent analyses (Akaike, 1974). The residual (co)variance structures of the selected models are presented in the APPENDIX (**Table A.1**). Prior to final analyses, assumptions of the residuals were evaluated using Studentized residuals

obtained from the final models. Normality was assessed using Shapiro-Wilk's test at $P > 0.05$ respecting the final model assumptions. In other words, residuals were evaluated within each HPS for gene-tissue combinations showing heterogeneous variances. When normality was not significant ($P < 0.05$), Studentized residuals with absolute values greater than 3 were removed one at a time until normality was met ($P > 0.05$). Levels of significant ($P < 0.05$) or trending ($0.05 < P < 0.10$) model terms were compared using Tukey's method and/or subjected to subsequent development of polynomial models (described below). All analyses were performed in SAS (version 9.4; SAS Institute, Cary, NC) using the GLIMMIX and UNIVARIATE procedures.

Polynomial models were tested and developed based on the final analysis of each gene-tissue combination. Within the overall model, orthogonal contrasts were written to test the linear and quadratic effects of HPS for the effects *H* in Eq. [1]. Similarly, orthogonal contrasts were constructed to test the linear, quadratic, and cubic effects of Met supplementation for effect of *Met(C)* in Eq. [1]. In all analyses, when higher-order effects were significant ($P < 0.05$), lower-order effects were included in the model to respect their orthogonality. After significant polynomial effects were determined, regression analyses were performed using data adjusted for the fixed effect of block while fixing the variance-covariance parameters obtained from the final model. Results are presented as $\log_2$ fold-change ($\log_2$FC). All analyses were performed in SAS (version 9.4; SAS Institute, Cary, NC) using the GLIMMIX procedure.

Results

The BW gain and feed intake of broilers fed graded levels of DL-Met during the grower phase (d 10 – 28) and challenged with LPS *in vivo* is shown in **Table 3.4**. The BW gain and feed intake of broilers increased ($P < 0.001$) for Met5 but was not significantly different from Met4

birds during d 0 – 24. During d 26 – 28, diets Met3 – Met5 showed increased ($P < 0.001$) BW gain and feed intake. The gain:feed of broilers fed graded levels of DL-Met during the grower phase (d 10 – 28) and challenged with LPS *in vivo* is shown in **Table 3.5**. During d 0 – 24 and 0 – 28, gain:feed increased ($P < 0.001$) for Met2 – Met5 birds. Diets Met2 – Met5 also had increased ($P < 0.001$) gain:feed during d 26 – 28, but Met2 was not significantly different from Met1. The LTSAA and Met0 birds were not significantly different for all gain:feed periods analyzed except for d 10 – 24.

The BW gain and gain:feed data were fitted to a 1-slope broken-line regression model to estimate the requirement of supplemental DL-Met (**Figures 3.2 & 3.3**). The requirement of supplemental DL-Met for BW gain and gain:feed before the *in vivo* LPS challenge from d 10 – 24 was estimated to be 0.18% (0.33% total Met) of the diet to reach a maximal level of 61 g/bird/d and 0.15% (0.30% total Met) of the diet to reach a maximal level of 660 g/kg, respectively. During the *in vivo* LPS challenge (d 24 – 26), the requirement for BW gain and gain:feed decreased numerically compared to before the *in vivo* LPS challenge and was estimated to be 0.17% (0.32% total Met) of the diet to reach a maximal level of 33 g/bird/d and 0.09% (0.24% total Met) of the diet to reach a maximal level of 344 g/kg, respectively. The growing period (d 26 – 28) following the *in vivo* LPS challenge was numerically higher than the requirement for supplemental DL-Met for BW gain and gain:feed both prior (d 10 – 24) to and during (d 24 – 26) the *in vivo* LPS challenge. The BW gain following (d 26 – 28) the *in vivo* LPS challenge was estimated to be 0.19% (0.34% total Met) of the diet to reach a maximal level of 76 g/bird/d while gain:feed was estimated to be 0.15% (0.30% total Met) of the diet to reach a maximal level of 697 g/kg.

The absolute liver DM weight and relative liver weight of broilers fed graded levels of DL-Met during the grower (d 10 – 28) phase and challenged with LPS *in vivo* are reported in **Table 3.6**. The absolute liver DM weight increased ($P < 0.001$) statistically and numerically at -24, 5, 24, and 72 HPS for Met4 but was not significantly different from some of the other DL-Met supplemented treatment groups at -24, 24, and 72 HPS. On a relative to BW basis, the liver DM weight was impacted ($P < 0.010$) by dietary treatment at -24, 5, and 72 HPS. Relative liver DM weight was not impacted ($P = 0.100$) at 24 HPS.

The body temperature of broilers fed graded levels of DL-Met during the grower (d 10 – 28) phase and challenged with LPS *in vivo* are shown in **Table 3.7**. There was no significant impact ($P > 0.05$) from dietary treatment on the body temperature of broilers at -24, 5, 24, and 72 HPS. However, when considering the main effect of HPS, the body temperature of broilers during an *in vivo* LPS challenge in the grower phase (d 10 – 28) was elevated ($P < 0.001$) at 5, 24, and 72 HPS (**Figure 3.4**).

All hematology data are reported in **Tables 3.8 – 3.10**. Hematocrit and total protein were impacted ($P < 0.012$) by dietary treatment at 24 and 72 HPS (**Table 3.8**). Heterophils and the ratio of heterophils to lymphocytes were impacted ($P < 0.005$) by dietary treatment at -24, 5, 24, and 72 HPS (**Table 3.9**). Additionally, lymphocytes were impacted ($P < 0.001$) by dietary treatment, but only at -24, 24, and 72 HPS. Monocyte/azurophil granules were impacted ($P = 0.016$) by dietary treatment at 72 HPS and eosinophils and basophils were impacted ($P < 0.047$) at -24 HPS (**Table 3.10**).

The impact of an *ex vivo* LPS stimulation on the IL-6 concentration of splenic macrophages isolated from broilers fed diets varying in supplemental DL-Met and challenged *in vivo* with LPS is reported in **Table 3.11.** Nearly all control samples yielded IL-6 concentrations

below the detectable limit of the ELISA kit (<0.085 ng/ml). Consequently, any data from control samples were removed from statistical analyses. There was no significant impact ($P = 0.241$) to IL-6 concentration by dietary treatment of splenic macrophages 6 h following an *ex vivo* stimulation with LPS.

Hepatic and splenic mRNA expression of *IL-1β* is reported in **Table 3.12**. Expression of hepatic and splenic *IL-1β* was independently impacted ($P < 0.042$) by HPS and supplemental L-cysteine. Dietary treatment and supplemental level of DL-Met independently impacted ($P < 0.009$) splenic expression of *IL-1β* but had no impact ($P > 0.05$) on hepatic expression of *IL-1β*. Dietary treatment or supplemental level of DL-Met differentially impacted ($P < 0.007$) expression of hepatic and splenic *IL-1β* depending on HPS. The interaction effect of HPS on supplemental L-cysteine had no impact ($P > 0.05$) on splenic *IL-1β* expression, while liver *IL-1β* expression was impacted ($P = 0.028$).

Hepatic and splenic mRNA expression of *IL-6* is reported in **Table 3.13**. The hepatic expression of *IL-6* was independently impacted ($P < 0.001$) by dietary treatment and supplemental L-cysteine, while splenic expression of *IL-6* was not impacted ($P < 0.05$). Both hepatic and splenic *IL-6* were impacted ($P < 0.001$) by HPS and not impacted ($P > 0.05$) by supplemental level of DL-Met. Dietary treatment differentially impacted ($P = 0.008$) expression of hepatic *IL-6* depending on HPS and was statistically highest for Met2 and Met4 with an FC value over 190 and 170, respectively, at 5 HPS. The interaction effect of dietary treatment or supplemental level of DL-Met on HPS had no impact ($P > 0.05$) on the splenic expression of *IL-6*. Supplemental level of DL-Met differentially impacted ($P = 0.016$) expression of hepatic *IL-6* depending on HPS. The interaction effect of HPS on supplemental L-cysteine had no impact ($P > 0.05$) on liver *IL-6* expression, while splenic *IL-6* expression was impacted ($P = 0.025$).

Hepatic and splenic mRNA expression of *IL-10* is reported in **Table 3.14**. Dietary treatment, HPS, and supplemental L-cysteine independently impacted ($P < 0.001$) hepatic and splenic expression of *IL-10*, and only hepatic expression of *IL-10* was independently impacted ($P = 0.018$) by the supplemental level of DL-Met. Dietary treatment or supplemental level of DL-Met did not differentially impact ($P > 0.05$) expression of hepatic and splenic *IL-10* depending on HPS. The interaction effect of HPS on supplemental L-cysteine had no impact ($P > 0.05$) on splenic and liver *IL-10* expression.

Regression plots of the log_2FC of each dietary treatment of liver and spleen mRNA expression of *IL-1β*, *IL-10*, and *IL-* are displayed in **Figure 3.5.**

Discussion

Although vital for overcoming infection, an activated immune system has associated metabolic costs and changes the partitioning of nutrients (Humphrey and Klasing, 2004; Iseri and Klasing, 2013). Evidence shows that diets containing higher levels of the amino acid Met are needed to optimize some immune performance parameters than what are required to maximize the growth response (Tsiagbe et al., 1987b; Swain and Johri, 2000; Rama Rao et al., 2003). In the current study, we investigated the impact of graded levels of dietary DL-Met during an *in vivo* acute immune challenge on growth and immune performance and the requirement for supplemental DL-Met for growth.

Dietary treatment had no impact on the body temperature of broilers before, during, or after an *in vivo* LPS challenge, but there was a main effect of HPS, such that birds injected i.p. with LPS had increased body temperature post-stimulation. This finding solidifies that the broilers were immunologically challenged and producing an immune response regardless of the level of supplemental DL-Met, as fever, which is a rise in core body temperature compared to the

normal body temperature range, is a key indicator of inflammation (El-Radhi et al., 2009; Evans et al., 2015). Additionally, the main effect of HPS on body temperature shows body temperature failed to return to the pre-challenge (i.e., -24 HPS) level by the end of the study, indicating birds may have still been experiencing immune-related impacts of the acute *in vivo* LPS challenge at study conclusion. Although a necessary component of an immune response, fever is metabolically costly (Evans et al., 2015). Indeed, the *in vivo* LPS challenge activated the immune system in our experiment, but the level of supplemental dietary Met has no impact on the febrile response or its amelioration within 72 HPS.

As expected, during the pre-challenge period (d 10 – 24), growth performance was improved with the addition of supplemental DL-Met to the diet. These findings are similar to those of Dilger and Baker (2007), which showed broiler growth performance increases when a sulfur amino acid-deficient purified or practical-type diet is supplemented with Met (Dilger and Baker, 2007). Broiler growth efficiency was decreased during the acute *in vivo* LPS challenge (d 24 - 26) by as much as 100% for the LTSAA birds when compared to the pre-challenge period (d 10 – 24). For most dietary treatments, BW gain was numerically decreased during the challenge while feed intake was marginally changed relative to the pre-challenge values, suggesting nutrients were not being prioritized as much for growth during the challenge. Interestingly, the requirement of supplemental DL-Met estimated from the 1-slope broken-line regression model was decreased during the immune challenge (d 24 – 26) for BW gain by about 7% and feed efficiency by roughly 39% compared to the pre-challenge period (d 10- 24). Klasing and Barnes (1988) also reported that the requirement for Met based on feed efficiency decreases during repeated acute immune challenges. The authors hypothesized that in non-challenged conditions for growing chicks, growth and tissue accretion make up the requirement for Met; however, upon

immune activation, nutrients are no longer prioritized for these processes and shift towards immune-supporting functions (Klasing and Barnes, 1988). Furthermore, the decrease in the requirement of Met for growth outweighs that of the increase in the requirement of Met for immune function during an immune challenge, resulting in a net decrease in the requirement for Met (Klasing and Barnes, 1988). This pattern was apparent in this experiment, as the requirement for supplemental DL-Met for BW gain and gain:feed decreased during the acute inflammatory *in vivo* challenge compared to pre-challenge.

Although the requirement of DL-Met decreased during the *in vivo* LPS challenge, during the recovery phase (d 26 – 28), the requirement for supplemental DL-Met for gain:feed (0.15%; 0.30% total Met) increased by over 70% compared to the estimated (0.09%; 0.24% total Met) supplemental DL-Met requirement during the *in vivo* LPS challenge (d 24 – 26) and was similar to the estimated (0.16%; 0.31% total Met) pre-challenge supplemental DL-Met requirement. Under non-challenged conditions, it is expected that the requirement for Met decreases as growing broilers age (Aviagen, 2019). However, our results show a similar requirement for supplemental DL-Met during the pre-challenge and recovery phase, suggesting broilers may have been undergoing compensatory gain post-challenge to account for losses in growth during the challenge.

Compensatory gain is an increase in efficiency compared to birds of similar age, and the nutrient profile of the diet can impact the extent of compensatory growth experienced by a bird (Zubair and Leeson, 1996). The period in which a bird can undergo compensatory gain and improve growth efficacy could be lost if insufficient amino acids are available (Klasing and Leshchinsky, 2000). Comparing the plateaus of the gain:feed regression models (i.e., level of feed efficiency at requirement) and results from the ANOVA on gain:feed also indicates the

broilers may have been undergoing a period of compensatory gain from the acute challenge. The feed efficiency of broilers during the recovery phase (d 26 – 28) was 697 g/kg, the highest feed efficiency reported during the regression analyses. The feed efficiency for the individual dietary treatments was numerically greater during the recovery phase (d 26 – 28) compared to their respective gain:feed during the pre-challenge phase (d 10 – 24), but only for DL-Met supplemented treatment groups (i.e., Met1 – Met5). This demonstrates the importance of DL-Met in promoting compensatory gain from acute immune-induced growth performance losses, which aids in efficiently restoring homeostasis and returning the bird to a typical growth curve trajectory.

The liver plays a crucial role in the rapid production of the acute phase, anti-microbial, and complement proteins needed to facilitate a robust, innate response to stimuli (Robinson et al., 2016). Following a challenge with non-infectious stimuli, chicks increase protein synthesis in the liver (Klasing and Austic, 1984). Additionally, immune activation by *E. coli* changed the weight of the liver and elicited expression of acute phase proteins, which represented a greater nutritional cost than the adaptive response itself (Iseri and Klasing, 2013). Our findings confirm the dynamic changes that occur in liver DM weight during an acute inflammatory challenge. At 5 HPS, birds receiving diets Met3 – Met5 had numerically increased liver DM weight while the remaining diets showed a numerical decrease relative to -24 HPS liver DM weights. Birds receiving diets Met3 – Met5 may have started responding to the LPS stimulation by initiating the acute phase response more swiftly than other dietary treatments. Additionally, rarely are liver weights reported on a DM basis; however, this removes water fluctuation as a factor for such drastic liver weight changes and accounts for alterations in weight that reflect changes in protein metabolism of the liver. In chickens, during an active acute phase response in the liver, acute

phase proteins can increase by 1- to 10- fold, and each maintains a distinct purpose in facilitating the response (Cray et al., 2009). By 24 HPS, a numeric increase in liver DM weight for every treatment was shown compared to its pre-challenge DM weight at -24 HPS, with Met5 increasing by over 54% (3.85 g). Uptake of additional sulfur amino acids from the diet may provide vital substrates needed by the liver for protein synthesis. In non-challenged conditions, the liver uptakes 70% and 17% of cysteine and Met, respectively, from the portal vein in pigs (Hou et al., 2020). Interestingly, liver DM relative to BW basis was not impacted by dietary treatment at 24 HPS. These findings suggest dietary supplemental DL-Met influences initiation of the acute phase response, but these changes occur in the immediate post-stimulation period.

Cytokine gene expression was examined to explore how alterations in dietary sulfur amino acid levels affect the immune response at a molecular level. All hepatic and splenic cytokine mRNA expression was significantly impacted by HPS, demonstrating the rapid transcription of these vital signal molecules upon immune activation. Cytokines, an integral part of overcoming infection, act as mediators to facilitate an immune response (Kogut, 2000). Hepatic *IL-6* mRNA expression showed the greatest response during the experiment as evidenced by the regression plots in which *IL-6* displayed the numerically highest $\log_2$FC, and at 5 HPS, Met2 and Met4 were increase by a fold-change of over 197 and 172, respectively, compared to the other time-points and dietary treatments. Moreover, an interaction effect between HPS and dietary treatment or supplemental level of DL-Met was displayed for hepatic mRNA expression of *IL-6*. The acute, rapid increase in FC of *IL-6* expression at 5 HPS further suggests initiation of the acute phase response, as *IL-6* signals to hepatocytes in the liver to alter the production of acute-phase proteins (Chamanza et al., 1999). Together the findings from the liver DM weights, relative liver DM weights, and hepatic *IL-6* expression data suggest the

dietary level of DL-Met is most influential during the onset of the innate response. Specifically, it may aid in the initiation of a robust acute phase response. More research is needed to corroborate these findings, as few other studies track cytokine gene expression during inflammatory challenges in broilers fed diets varying in levels of Met. Further, these may be impacted by the source of Met as well.

As a hallmark indicator of inflammation, *IL-1β* was impacted by HPS in the liver and spleen, demonstrating the responsiveness of both organs to the *in vivo* LPS challenge. This result is similar to that reported by Tan et al. (2014), where splenic *IL-1β* expression was impacted by an *in vivo* challenge to LPS (Tan et al., 2014). Expression of *IL-1β* was the only cytokine to display an interaction effect of dietary treatment or supplemental level of DL-Met on HPS for both the liver and spleen. These results are consistent with Takahashi et al. (1997), who found plasma IL-1-like activity is altered in response to an *in vivo* LPS stimulation with diets deficient or sufficient in TSAA (Takahashi et al., 1997). Klasing and Barnes (1988) found that the level of Met in the diet impacts serum IL-1-like activity in response to an *in vivo* LPS stimulation (Klasing and Barnes, 1988). In the present study, splenic *IL-1β* expression, but not hepatic, was significantly impacted by the level of supplemental DL-Met within diets Met0 – Met5. The spleen is a secondary lymphoid organ where antigen, in this case, LPS, processing by lymphocytes occurs (Lewis et al., 2019). The T-lymphocytes are mediators of the adaptive response, and their activity can be impacted by IL-1 cytokines (Santarlasci et al., 2013). While the level of dietary Met may influence T-lymphocyte activity, this experiment was targeted at investigating the innate immune response and concluded at 72 HPS. Further research is needed to test whether changes also occur during the adaptive immune response.

The anti-inflammatory cytokine IL-10 down-regulates the immune response to various stimuli, effectively ameliorating damage to tissues incurred during an active immune response (Couper et al., 2008; Saraiva and O'Garra, 2010). Expression of hepatic and splenic *IL-10* was not impacted by the interaction of dietary treatment or level of supplemental DL-Met on HPS, despite HPS independently impacting both hepatic and splenic expression of *IL-10.* These results are similar to those found by Rasheed et al. (2020), in which challenged (*Eimeria*) versus non-challenged birds displayed increased expression of cecal *IL-10*; however, within challenged birds, the main effect of a sulfur-containing compound, methylsulfonylmethane, had no impact (Rasheed et al., 2020). Interestingly, within Met0 – Met5 birds, supplemental DL-Met linearly impacted the expression of hepatic *IL-10* expression. Authors Liu et al. (2019) found comparable results by subjecting birds to chronic heat stress and found that expression of hepatic *IL-10* is impacted when 100% or 130% of the TSAA requirement (AMINOChick 2.0; Evonik Nutrition and Care GmbH, Hanau, Germany) is fed (Liu et al., 2019). The expression of *IL-10* is altered in the spleen and liver during an *in vivo* LPS challenge in broilers, but the supplemental level of DL-Met had little influence during the anti-inflammatory innate immune response of these birds. These data suggest that the level of Met in the diet is a more potent regulator of the pro-inflammatory response rather than the anti-inflammatory response.

Dietary treatment did not influence IL-6 production of primary splenic macrophages stimulated *ex vivo* with LPS and isolated from broilers fed graded levels of DL-Met and challenged *in vivo* with LPS. A few factors may have impacted our ability to uncover differences in IL-6 production from splenic macrophages. The technique used to isolate and culture macrophages from the spleen may not have allowed the cells to culture for an adequate amount of time to promote a homogenous macrophage population. No analysis was conducted during the

experiment to identify the population of cells cultured. Nevertheless, isolation and culture of primary monocytes from chicken blood by gradient centrifugation similar to the technique utilized in this experiment have been validated by flow cytometry elsewhere (Verwoolde et al., 2020). Following overnight adhesion and an additional 24 h of culture, the population of macrophages was about 10-20%, while 7 d after culture, the population of macrophages was roughly 90-100% (Verwoolde et al., 2020). Our technique only allowed the splenic cells to culture for 6 h, perhaps limiting the number of cultured macrophages. The cultures used here could have contained populations of cells that respond differently from macrophages to LPS. Another possible explanation takes into consideration the impact of genetic selection on the immunity of a broiler. Over the past 60 years, it is well known that broilers have been genetically selected for desirable growth traits to enhance efficiency, amounting to an increase of growth by more than 400% (Zuidhof et al., 2014).

Although critical to the sustainability of the industry and the ability to meet the growing demand of consumers for poultry meat, genetic selection for growth in broilers may be at the expense of immunity (Han and Smyth, 1972; Swaggerty et al., 2019). In addition, the functionality of macrophages differs between commercial genetic lines of broilers (Qureshi and Miller, 1991). Lastly, compared to layers, broiler splenic cells stimulated with LPS have a lessened response of the IL-6 related pro-inflammatory cytokine myelomonocytic growth factor (Leshchinsky and Klasing, 2001). Additionally, splenic *IL-6* expression from the *in vivo* challenge was not significantly impacted by the level of supplemental DL-Met within diets Met0 – Met5. Moreover, splenic *IL-1β* expression was significantly impacted by the level of supplemental DL-Met within diets Met0 – Met5. It is plausible that analysis of a different

cytokine, modification of the culture technique, or using a different genetic line of broilers would yield different results because of the previously described factors impacting macrophage activity.

Much like the avian macrophage, leukocytes (i.e., white blood cells) are also essential to innate immunity. Lymphocytes and heterophils make up over 90% of leukocytes, and the ratio of heterophils to lymphocytes (H:L) can be used as an indicator of stress in avian species (Gross and Siegel, 1983; Davis et al., 2008; Minias, 2019). Heterophils can ward off disease by discharging anti-microbial peptide-containing granules called gallinacins (i.e., mammalian β-defensins) (Genovese et al., 2013). Gallinacins are abundant in cysteine as their structure contains 6 cysteine invariant residues connected by 3 intramolecular disulfide linkages and possess anti-microbial properties specifically towards Gram-negative bacteria (Harwig et al., 1994; Harmon, 1998; Sugiarto and Yu, 2004). Dietary treatments low in sulfur amino acids displayed increased H:L ratio upon stimulation, while supplemental DL-Met treatments at 5, 24, and 72 HPS containing at least Met3 or above had decreased H:L ratios. Access to an adequate amount of sulfur amino acids during an acute inflammatory challenge may support heterophil efficacy by providing additional substrates to produce vital cysteine-rich anti-microbial peptides capable of destroying Gram negative bacteria. Additionally, the Met3 diet contained a level of supplemental DL-Met (0.216% of the diet) numerically higher than any estimated requirements post-challenge for supplemental DL-Met from the 1-slope broken-line regression model for both BW gain and gain:feed. Although the hematology data was not fitted to the same 1-slope broken-line regression model, because at least Met3 or higher was required to reduce the H:L ratio post-stimulation, the associated requirement for supplemental DL-Met to lower stress and influence heterophil activity as measured by the H:L ratio would be expected to be greater than the level of supplemental DL-Met in Met3. Other studies have also found the required level of dietary Met

for immune performance and growth performance differ. Some studies have demonstrated a greater level of Met was required to optimize the antibody response compared to growth (Tsiagbe et al., 1987a; b; Rama Rao et al., 2003). An enhanced cellular response also tends to require more dietary Met relative to growth maximization (Tsiagbe et al., 1987a; b; Swain and Johri, 2000; Rama Rao et al., 2003). Altogether, supplemental DL-Met influences avian heterophil activity while fighting off pathogens, and the level of supplemental DL-Met required to reduce stress as indicated by the H:L ratio may be greater than what is needed to maximize growth.

In conclusion, graded levels of supplemental DL-Met fed to broilers during an *in vivo* challenge to LPS impacts growth and immune performance and the requirement for supplemental DL-Met. Growth performance was improved with the addition of supplemental DL-Met before, during, and after an *in vivo* LPS challenge. The estimated requirement for supplemental DL-Met numerically decreased for BW gain and gain:feed during (d 24 – 26) the *in vivo* LPS challenge by about 7% and 39%, respectively, compared to the pre-challenge period (d 10- 24). Liver DM weight was impacted by dietary treatment at all time-points. However, on a relative to weight basis, liver DM weight is no longer impacted by dietary treatment at 24 HPS. Furthermore, hepatic and splenic cytokine mRNA expression was impacted. Specifically, liver expression of *IL-6* was differentially impacted by HPS on the supplemental level of DL-Met and was of the greatest magnitude at 5 HPS. Dietary treatment of graded levels of DL-Met is also influential to heterophil activity, thereby changing the H:L ratio during an *in vivo* LPS challenge. More supplemental DL-Met was required to reduce the ratio of H:L compared to what was required to maximize growth during the challenge (d 24 – 26). Splenic macrophage IL-6 production to an *ex vivo* response was not impacted by the level of supplemental DL-Met fed to

the broilers from which the cells were isolated, although modifications of culture technique and cytokine analysis may yield different results. Taken together, the findings from this experiment suggest that impacts made by the amino acid Met occur during the onset of the innate response. More specifically, it may support the initiation of a robust acute phase response within the liver. The activity of heterophils was influenced by the level of supplemental DL-Met such that more supplemental DL-Met was required to reduce stress as measured by the H:L ratio than what was required for growth. Further research is needed to elucidate whether alterations in the innate immunity of broilers made by Met supports broiler health up to market age. Additionally, because multiple products are available to broiler producers as a source of Met in the diet, future studies that include Met source as a variable are also highly valuable in establishing the relationship between broiler health and Met to provide the most meaningful impact to broiler producers.

Tables and Figures

Table 3.1. Ingredient composition of the starter (d 0 – 10) diet[1]

Item, % as-fed	Level
Corn	50.09
Soybean meal	40.90
Soy oil	4.00
Sodium chloride	0.40
Limestone	1.20
Dicalcium phosphate	2.10
Vitamin premix[2]	0.20
Mineral premix[3]	0.15
Choline chloride	0.32
L-Lys HCl	0.18
DL-Met	0.38
L-Thr	0.09
Calculated composition	
AME_n, kcal/kg	2949
CP	22.48
Lys	1.43
Met + Cys	1.04
Thr	0.96
Ca	1.06
Non-phytate P	0.49
Analyzed composition	
CP	22.81
Lys	1.41
Met + Cys	1.02
Thr	0.95

[1]Abbreviations: AME_n, nitrogen-corrected apparent metabolizable energy.
[2]Provided per kilogram of complete diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 μg; dl-α-tocopheryl acetate, 11 IU; vitamin B12, 0.01 mg; riboflavin, 4.41 mg; d-Ca-pantothenate, 10 mg; niacin, 22 mg; and menadione sodium bisulfite, 2.33 mg.
[3]Provided as milligrams per kilogram of complete diet: Mn, 75 from MnO; Fe, 75 from $FeSO_4 \cdot 7H_2O$; Zn, 75 from ZnO; Cu, 5 from $CuSO_4 \cdot 5H_2O$; I, 0.75 from ethylene diamine dihydroiodide; and Se, 0.1 from Na_2SeO_3.

Table 3.2. Ingredient composition of the experimental grower (d 10 – 28) diets

	Dietary treatment						
Ingredient, % as-fed	LTSAA	Met0	Met1	Met2	Met3	Met4	Met5
Corn	31.61	31.61	31.61	31.61	31.61	31.61	31.61
Soybean meal	16.57	16.57	16.57	16.57	16.57	16.57	16.57
Dextrose	31.47	31.11	31.04	30.97	30.89	30.82	30.75
Cellulose	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Soybean oil	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Sodium bicarbonate	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Limestone	1.03	1.03	1.03	1.03	1.03	1.03	1.03
Dicalcium phosphate	2.17	2.17	2.17	2.17	2.17	2.17	2.17
Vitamin premix[1]	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Mineral premix[2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Choline chloride	0.37	0.37	0.37	0.37	0.37	0.37	0.37
Potassium carbonate	0.78	0.78	0.78	0.78	0.78	0.78	0.78
Amino acid mixture[3]	7.65	7.65	7.65	7.65	7.65	7.65	7.65
DL-Met	0.00	0.00	0.07	0.14	0.22	0.29	0.36
L-cysteine	0.00	0.36	0.36	0.36	0.36	0.36	0.36

[1]Provided per kilogram of complete diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 μg; dl-α-tocopheryl acetate, 11 IU; vitamin B12, 0.01 mg; riboflavin, 4.41 mg; d-Ca-pantothenate, 10 mg; niacin, 22 mg; and menadione sodium bisulfite, 2.33 mg.

[2]Provided as milligrams per kilogram of complete diet: Mn, 75 from MnO; Fe, 75 from $FeSO_4 \cdot 7H_2O$; Zn, 75 from ZnO; Cu, 5 from $CuSO_4 \cdot 5H_2O$; I, 0.75 from ethylene diamine dihydroiodide; and Se, 0.1 from Na_2SeO_3.

[3]Provided as % of complete diet: 0.69 L-arginine; 0.11 L-histidine; 0.37 L-isoleucine; 0.40 L-leucine; 0.74 L-lysine HCL; 0.28 L-phenylalanine; 0.22 L-tyrosine; 0.47 L-threonine; 0.09 L-tryptophan; 0.44 L-valine; 0.33 L-serine; 0.58 L-glycine; 0.43 L-proline; 0.79 L-aspartic acid; 1.36 L-glutamic acid; 0.36 L-alanine.

Table 3.3. Calculated and analyzed nutrient composition of the experimental grower (d 10 – 28) diets[1]

	Dietary treatment						
Item, % as-fed	LTSAA	Met0	Met1	Met2	Met3	Met4	Met5
Calculated composition							
AME_n, kcal/kg	3146	3141	3141	3141	3141	3141	3141
CP	16.71	16.98	17.02	17.06	17.10	17.14	17.19
Lys	1.13	1.13	1.13	1.13	1.13	1.13	1.13
Met	0.15	0.15	0.22	0.29	0.36	0.44	0.51
Cys	0.15	0.51	0.51	0.51	0.51	0.51	0.51
Met + Cys	0.30	0.66	0.73	0.81	0.88	0.95	1.02
Thr	0.85	0.85	0.85	0.85	0.85	0.85	0.85
Ca	0.93	0.93	0.93	0.93	0.93	0.93	0.93
Non-phytate P	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Analyzed composition							
CP	16.85	16.84	17.02	17.13	17.26	17.18	17.11
Met	0.15	0.15	0.21	0.28	0.36	0.41	0.50
Supp. DL-Met[2]	<0.01	<0.01	0.06	0.13	0.21	0.28	0.34
Cys	0.16	0.45	0.47	0.46	0.48	0.46	0.46
Met + Cys	0.30	0.60	0.68	0.74	0.83	0.86	0.96
Lys	1.08	1.11	1.06	1.04	1.07	1.06	1.04
Thr	0.80	0.80	0.79	0.78	0.80	0.77	0.79

[1]Abbreviations: AME_n, nitrogen-corrected apparent metabolizable energy; supp., supplemental.
[2]Supplemental DL-Met refers to the analyzed level of supplemental DL-Met found in the diet and does not include Met from intact protein sources (i.e., corn and soybean meal).

Table 3.4. Body weight gain and feed intake of broilers fed graded levels of DL-Met during the grower (d 10 – 28) phase and challenged with LPS[1]

Item	Body weight gain, g/bird/d						Feed intake, g/bird/d					
Diet Phase	10-24	0-24	24-26	26-28	24-28	0-28	10-24	0-24	24-26	26-28	24-28	0-28
LTSAA	12^{d}	17^{e}	0^{e}	10^{d}	5^{e}	16^{e}	45^{e}	38^{e}	47^{e}	41^{d}	45^{d}	39^{d}
Met0	9^{e}	16^{e}	4^{e}	7^{d}	6^{e}	14^{e}	42^{e}	36^{e}	50^{e}	33^{d}	43^{d}	37^{d}
Met1	25^{c}	25^{d}	17^{d}	29^{c}	23^{d}	25^{d}	56^{d}	44^{d}	59^{d}	61^{c}	60^{c}	46^{c}
Met2	51^{b}	40^{c}	29^{bc}	61^{b}	46^{c}	41^{c}	82^{c}	59^{c}	86^{c}	95^{b}	90^{b}	62^{b}
Met3	60^{a}	45^{b}	27^{c}	73^{a}	50^{bc}	46^{b}	90^{b}	64^{b}	93^{bc}	106^{a}	98^{a}	67^{a}
Met4	61^{a}	46^{ab}	34^{ab}	77^{a}	56^{ab}	48^{ab}	92^{ab}	65^{ab}	100^{a}	112^{a}	105^{a}	69^{a}
Met5	62^{a}	47^{a}	40^{a}	78^{a}	57^{a}	49^{a}	95^{a}	67^{a}	95^{ab}	110^{a}	102^{a}	70^{a}
Pooled SEM	0.76	0.52	2.29	2.98	2.28	0.71	1.49	0.94	2.47	3.61	2.58	1.01
Overall model	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a-e]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are least-square means initially derived from 10 birds per replicate cage, 7 replicate cages per dietary treatment. The average body weight at study initiation was 43 ± 4 g. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 hours post-stimulation). Abbreviations: LPS; lipopolysaccharide; SEM, standard error of the mean.

Table 3.5. Gain:feed ratio of broilers fed graded levels of DL-Met during the grower (d 10 – 28) phase and challenged with LPS[1]

Item		Gain:feed, g/kg					
Diet	Phase	10-24	0-24	24-26	26-28	24-28	0-28
LTSAA		253[d]	454[c]	0[c]	205[c]	51[c]	405[c]
Met0		213[e]	439[c]	92[c]	201[c]	57[c]	386[c]
Met1		442[c]	565[b]	286[b]	483[b]	170[b]	538[b]
Met2		627[b]	682[a]	337[ab]	647[ab]	221[ab]	664[a]
Met3		665[a]	709[a]	296[b]	694[a]	219[ab]	689[a]
Met4		663[a]	704[a]	342[a]	690[a]	230[a]	692[a]
Met5		653[ab]	706[a]	408[a]	706[a]	238[a]	701[a]
Pooled SEM		7.19	7.08	26.01	27.55	9.54	11.24
Overall Model		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a-e]Means without a common superscript letter differ within a column ($P < 0.05$).
[1]Values are least-square means initially derived from 10 birds per replicate cage, 7 replicate cages per dietary treatment. The average body weight at study initiation was 43 ± 4 g. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 hours post-stimulation). Abbreviations: LPS; lipopolysaccharide; SEM, standard error of the mean.

Table 3.6. Absolute liver weight and relative liver weight of broilers fed graded levels of DL-Met during the grower (d 10 – 28) phase and challenged with LSP[1]

	Item	Absolute liver DM weight, g				Relative liver DM weight, %[2]			
Diet	HPS	-24	5	24	72	-24	5	24	72
LTSAA		3.43^{d}	2.99^{d}	3.80^{d}	3.78^{d}	0.73^{ab}	0.69^{b}	0.84	0.80^{b}
Met0		3.64^{d}	3.47^{d}	3.82^{d}	4.20^{d}	0.82^{a}	0.77^{a}	0.94	1.05^{a}
Met1		4.97^{c}	4.81^{c}	6.55^{c}	5.82^{c}	0.78^{a}	0.72^{ab}	0.98	0.77^{bc}
Met2		7.87^{ab}	6.91^{b}	10.25^{b}	9.93^{ab}	0.76^{a}	0.71^{b}	0.98	0.80^{b}
Met3		6.91^{b}	7.36^{b}	10.32^{ab}	10.25^{ab}	0.60^{c}	0.61^{c}	0.83	0.76^{bc}
Met4		8.23^{a}	8.66^{a}	11.66^{a}	11.10^{a}	0.71^{abc}	0.70^{b}	0.93	0.86^{b}
Met5		7.01^{ab}	7.34^{b}	10.86^{ab}	9.78^{b}	0.64^{bc}	0.61^{c}	0.88	0.68^{c}
Pooled SEM		0.48	0.48	0.49	0.47	0.04	0.03	0.04	0.04
Overall Model		<0.001	<0.001	<0.001	<0.001	0.010	<0.001	0.100	<0.001

[a-d]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are least-square means initially derived from 1 bird per replicate, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 HPS). Abbreviations: LPS; lipopolysaccharide; HPS, host post-stimulation; SEM, standard error of the mean.

[2]Values were calculated by taking the weight of the organ (g) on a dry matter basis, dividing it by the body weight of the bird (g), and multiplying it by 100 to represent a percentage of body weight.

Table 3.7. Body temperature of broilers fed graded levels of DL-Met during the grower (d 10 – 28) phase and challenged with LPS[1]

	Hours post-stimulation			
Diet	-24	5	24	72
LTSAA	40.01	40.86	40.33	40.67
Met0	39.47	40.56	39.87	40.54
Met1	39.49	41.14	40.36	40.26
Met2	40.17	40.39	40.20	40.51
Met3	40.29	40.47	40.31	40.54
Met4	40.27	40.27	40.56	40.51
Met5	39.86	40.36	40.89	40.21
Pooled SEM	0.32	0.23	0.22	0.18
Overall model	0.331	0.107	0.088	0.481

[1]Values are least-square means initially derived from 1 bird per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 hours post-stimulation). Abbreviations: LPS; lipopolysaccharide; SEM, standard error of the mean.

Table 3.8. Hematocrit, total protein, and band heterophils of broilers fed graded levels of DL-Met during the grower (d 10 – 28) phase and challenged with LPS[1]

	Item	Hematocrit, %				Total protein, g/dL				Band heterophils, % of WBC			
Diet	HPS	-24	5	24	72	-24	5	24	72	-24	5	24	72
LTSAA		32^{abc}	29	27^{d}	27^{c}	3.4	2.7	3.0^{d}	3.4^{c}	0	3^{cd}	0	1^{bc}
Met0		34^{ab}	29	31^{abc}	30^{ab}	3.2	2.7	3.3^{cd}	3.8^{ab}	0	2^{cd}	0	0^{c}
Met1		34^{a}	29	32^{ab}	31^{ab}	3.5	2.9	3.5^{abc}	3.6^{bc}	0	4^{cd}	0	0^{c}
Met2		30^{c}	28	32^{a}	32^{a}	3.3	2.8	3.6^{abc}	3.9^{a}	0	2^{d}	0	1^{a}
Met3		31^{bc}	27	30^{bc}	31^{ab}	3.3	2.9	3.6^{ab}	3.8^{ab}	0	5^{bc}	0	1^{abc}
Met4		32^{bc}	27	30^{abc}	30^{ab}	3.5	2.8	3.3^{bcd}	3.8^{ab}	0	7^{b}	0	2^{ab}
Met5		31^{c}	27	29^{cd}	29^{b}	3.2	2.9	3.7^{a}	3.7^{ab}	0	10^{a}	0	2^{ab}
Pooled SEM		0.85	1.14	0.77	0.89	0.09	0.09	0.12	0.10	.[2]	1.08	.[2]	0.65
Overall Model		0.022	0.656	<0.001	0.005	0.255	0.582	<0.001	0.012	.[2]	<0.001	.[2]	0.023

[a-d]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are least-square means initially derived from 1 bird per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 HPS). Abbreviations: LPS; lipopolysaccharide; HPS, hours post-stimulation; WBC, white blood cells; SEM, standard error of the mean.

[2]Analysis was not performed due to all treatment values near equal or infinite likelihood.

Table 3.9. Heterophils, lymphocytes, and the ratio of heterophils to lymphocytes of broilers fed graded levels of DL-Met during the grower (d 10 – 28) phase and challenged with LPS[1]

Item	Heterophils, % of WBC				Lymphocytes, % of WBC				Heterophils:lymphocytes[2]			
Diet HPS	-24	5	24	72	-24	5	24	72	-24	5	24	72
LTSAA	57^{a}	71^{abc}	57^{bc}	50^{ab}	25^{d}	18	30^{bcd}	37^{c}	2.5^{a}	5.9^{abc}	2.7^{bc}	1.5^{bc}
Met0	53^{a}	75^{ab}	71^{a}	60^{a}	29^{cd}	15	16^{e}	19^{d}	1.4^{b}	6.7^{ab}	4.9^{a}	3.3^{a}
Met1	45^{ab}	79^{a}	61^{ab}	47^{bc}	40^{bc}	11	24^{de}	34^{cd}	1.4^{b}	8.6^{a}	3.6^{ab}	1.6^{bc}
Met2	30^{c}	67^{bc}	60^{ab}	50^{ab}	48^{ab}	25	25^{cde}	24^{d}	0.7^{b}	3.0^{cd}	2.6^{bc}	2.1^{b}
Met3	35^{bc}	68^{bc}	50^{bcd}	30^{de}	56^{a}	19	38^{abc}	50^{ab}	0.7^{b}	4.9^{bcd}	1.2^{cd}	0.7^{d}
Met4	43^{abc}	55^{d}	46^{cd}	40^{cd}	47^{ab}	24	43^{ab}	42^{bc}	1.1^{b}	2.4^{d}	1.3^{cd}	1.0^{cd}
Met5	34^{bc}	63^{cd}	40^{d}	23^{e}	54^{ab}	21	45^{a}	60^{a}	0.8^{b}	2.8^{cd}	1.0^{d}	0.5^{d}
Pooled SEM	5.16	4.09	4.57	4.84	5.02	3.86	4.77	5.85	0.31	1.31	0.57	0.33
Overall Model	0.004	0.002	<0.001	<0.001	<0.001	0.152	<0.001	<0.001	<0.001	0.005	<0.001	<0.001

[a-e]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are least-square means initially derived from 1 bird per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 HPS). Abbreviations: LPS; lipopolysaccharide; HPS, hours post-stimulation; WBC, white blood cells; SEM, standard error of the mean.

[2]Values were calculated by taking heterophils as a percentage of white blood cells and dividing it by lymphocytes as a percentage of white blood cells to represent a ratio.

Table 3.10. Monocyte/azurophil granules, eosinophils, and basophils of broilers fed graded levels of DL-Met during the grower (d 10 – 28) phase and challenged with LPS[1]

	Item	Mono/azuro granules, % of WBC				Eosinophils, % of WBC				Basophils, % of WBC			
Diet	HPS	-24	5	24	72	-24	5	24	72	-24	5	24	72
LTSAA		2	3	2	2^{c}	7^{b}	2	4	7	9^{a}	3	6	4
Met0		3	2	2	3^{bc}	6^{bc}	1	6	8	6^{ab}	5	5	10
Met1		4	3	3	2^{bc}	4^{bc}	0	6	6	6^{ab}	3	7	6
Met2		3	2	1	6^{a}	13^{a}	1	6	8	3^{b}	3	7	7
Met3		3	2	2	4^{ab}	2^{c}	1	3	5	4^{b}	4	6	10
Met4		3	3	1	4^{abc}	4^{bc}	2	2	5	4^{b}	4	6	8
Met5		3	4	2	3^{bc}	4^{bc}	0	5	2	4^{b}	3	7	6
Pooled SEM		0.85	1.00	0.55	0.95	1.60	0.47	1.27	2.19	1.50	1.00	1.16	2.31
Overall Model		0.641	0.695	0.506	0.016	<0.001	0.050	0.200	0.104	0.047	0.600	0.617	0.191

[a-c]Means without a common superscript letter differ within a column ($P < 0.05$).

[1]Values are least-square means initially derived from 1 bird per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 HPS). Abbreviations: LPS; lipopolysaccharide; HPS, hours post-stimulation; WBC, white blood cells; SEM, standard error of the mean.

Table 3.11. The concentration of splenic macrophage-derived IL-6 six h post *ex vivo* stimulation with LPS isolated from broilers fed graded levels of DL-Met during the grower phase (d 10 – 28) and challenged *in vivo* with LPS[1]

	Stimulation	
Diet	Control	LPS
LTSAA	BDL	0.745
Met0	BDL	1.486
Met1	BDL	1.583
Met2	BDL	0.937
Met3	BDL	0.984
Met4	BDL	1.265
Met5	BDL	1.357
Pooled SEM	-	0.327
Overall model	-	0.241

[1]Values are reported as IL-6 (ng/ml) concentration and are least-square means initially derived from 2 wells per bird (i.e., 1 control well + 1 LPS well), 1 bird per replicate, and 7 replicates per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 hours post-stimulation). Splenic macrophages were isolated on d 26 and left to adhere overnight. On d 27, splenic macrophages were stimulated with a control RPMI-based treatment or LPS treatment at 100 ug/ml/well for 6 h. At which point, cell supernatants were removed, stored, and later analyzed for IL-6 concentration by an enzyme-linked immunosorbent assay kit. Abbreviations: LPS, lipopolysaccharide; BDL, below detectable limit; SEM, standard error of the mean.

Table 3.12. Liver and splenic mRNA expression of interleukin-1β from broilers fed graded levels of DL-Met during the grower phase (d 10 – 28) and challenged with LPS[1]

	Tissue	Liver			Spleen		
Treatment	HPS	-24	5	24	-24	5	24
LTSAA		0.60[a]	2.42[abcdef]	4.36[cdef]	1.26[abcdefg]	2.62[fgh]	2.14[defgh]
		[0.40; 0.90]	[1.31; 4.47]	[2.48; 7.68]	[0.84; 1.87]	[1.76; 3.90]	[1.44; 3.18]
Met0		1.00[ab]	3.76[bcdef]	9.78[f]	1.00[abcdef]	4.32[h]	1.92[cdefgh]
		[0.67; 1.49]	[2.04; 6.95]	[5.33; 17.95]	[0.67; 1.49]	[2.91; 6.42]	[1.29; 2.86]
Met1		1.15[abc]	2.92[bcdef]	2.29[abcdef]	1.14[abcdefg]	4.48[h]	0.76[abcd]
		[0.77; 1.72]	[1.58; 5.40]	[1.30; 4.03]	[0.77; 1.70]	[3.01; 6.66]	[0.51; 1.12]
Met2		1.17[abc]	6.62[ef]	3.06[bcdef]	0.48[a]	2.32[fgh]	0.99[abcdef]
		[0.78; 1.75]	[3.58; 12.24]	[1.74; 5.38]	[0.33; 0.72]	[1.56; 3.44]	[0.67; 1.48]
Met3		1.45[abcd]	4.70[def]	1.97[abcde]	0.62[ab]	1.87[cdefgh]	1.68[bcdefgh]
		[0.97; 2.17]	[2.54; 8.69]	[1.12; 3.47]	[0.40; 0.95]	[1.26; 2.78]	[1.09; 2.58]
Met4		1.48[abcd]	4.78[def]	2.40[abcdef]	0.69[abc]	2.92[gh]	1.24[abcdefg]
		[0.99; 2.22]	[2.59; 8.84]	[1.36; 4.23]	[0.46; 1.02]	[1.96; 4.34]	[0.83; 1.84]
Met5		1.04[abc]	3.47[bcdef]	4.40[cdef]	0.81[abcde]	2.21[efgh]	1.74[bcdefgh]
		[0.70; 1.56]	[1.88; 6.41]	[2.50; 7.74]	[0.54; 1.20]	[1.49; 3.28]	[1.17; 2.59]

Variance source	df	*P* – value	
Treatment	6	0.101	0.004
HPS	2	<0.001	<0.001
Linear effect	1	<0.001	0.130
Quadratic effect	1	<0.001	<0.001
HPS × Treatment	12	0.002	0.005
L-Cysteine	1	0.042	0.024
HPS × L-Cysteine	2	0.028	0.093
Supp. Met	5	0.226	0.009
Linear effect	1	-[2]	0.065
Quadratic effect	1	-[2]	0.002
Cubic effect	1	-[2]	0.399
HPS × Supp. Met	10	0.005	0.007

[a-h]Means without a common superscript letter differ within a tissue ($P < 0.05$).

[1]Analyses were performed as $\log_2$ relative expression, but values are reported as fold-change (95% confidence interval [lower limit; upper limit]) and are least-square means initially derived from 1 bird per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 HPS). Abbreviations: LPS, lipopolysaccharide; HPS, hours post-stimulation; supp., supplemental.

[2]*P* – value was not calculated because the higher-order effect was not significant ($P > 0.05$).

Table 3.13. Liver and splenic mRNA expression of interleukin-6 from broilers fed graded levels of DL-Met during the grower phase (d 10 – 28) and challenged with LPS[1]

	Tissue	Liver			Spleen		
Treatment	HPS	-24	5	24	-24	5	24
LTSAA		0.43[a]	14.76[de]	2.10[abc]	0.41	20.75	1.97
		[0.21; 0.86]	[7.38; 29.54]	[1.05; 4.20]	[0.25; 0.70]	[9.22; 46.69]	[1.22; 3.17]
Met0		1.00[abc]	33.41[ef]	3.26[bcd]	1.00	34.74	2.35
		[0.50; 2.00]	[16.69; 66.85]	[1.63; 6.53]	[0.59; 1.68]	[15.44; 78.17]	[1.46; 3.78]
Met1		0.88[abc]	37.42[ef]	3.15[bcd]	0.49	36.35	0.80
		[0.44; 1.77]	[18.70; 74.88]	[1.57; 6.29]	[0.29; 0.82]	[16.15; 81.79]	[0.50; 1.29]
Met2		0.65[ab]	197.98[f]	4.00[cd]	0.96	22.80	0.98
		[0.32; 1.30]	[98.94; 396.18]	[2.00; 8.01]	[0.57; 1.61]	[10.13; 51.30]	[0.61; 1.57]
Met3		1.63[abc]	59.68[ef]	2.94[bcd]	0.94	19.28	1.28
		[0.82; 3.26]	[29.83; 119.43]	[1.47; 5.88]	[0.56; 1.58]	[8.57; 43.4]	[0.79; 2.05]
Met4		1.73[abc]	172.23[f]	2.33[abc]	0.95	26.78	1.55
		[0.86; 3.46]	[86.07; 344.65]	[1.17; 4.67]	[0.57; 1.60]	[11.90; 60.26]	[0.96; 2.49]
Met5		1.81[abc]	78.23[ef]	3.16[bcd]	1.15	22.56	2.72
		[0.91; 3.63]	[39.10; 156.55]	[1.58; 6.33]	[0.68; 1.94]	[10.02; 50.76]	[1.69; 4.39]

Variance source	df	*P* – value	
Treatment	6	0.001	0.131
HPS	2	<0.001	<0.001
Linear effect	1	0.086	<0.001
Quadratic effect	1	<0.001	<0.001
HPS × Treatment	12	0.008	0.117
L-Cysteine	1	<0.001	0.224
HPS × L-Cysteine	2	0.062	0.025
Supp. Met	5	0.178	0.135
Linear effect	1	-[2]	-[2]
Quadratic effect	1	-[2]	-[2]
Cubic effect	1	-[2]	-[2]
HPS × Supp. Met	10	0.016	0.374

[a-f]Means without a common superscript letter differ within a tissue ($P < 0.05$).

[1]Analyses were performed as $\log_2$ relative expression, but values are reported as fold-change (95% confidence interval [lower limit; upper limit]) and are least-square means initially derived from 1 bird per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 HPS). Abbreviations: LPS, lipopolysaccharide; HPS, hours post-stimulation; supp., supplemental.

[2]*P* – value was not calculated because the higher-order effect was not significant ($P > 0.05$).

Table 3.14. Liver and splenic mRNA expression of interleukin-10 from broilers fed graded levels of DL-Met during the grower phase (d 10 – 28) and challenged with LPS[1]

	Tissue	Liver			Spleen		
Treatment	HPS	-24	5	24	-24	5	24
LTSAA		0.32	9.94	4.41	0.55	5.77	0.86
		[0.22; 0.49]	[5.66; 17.46]	[1.64; 10.43]	[0.37; 0.82]	[3.20; 10.42]	[0.48; 1.55]
Met0		1.00	21.29	10.60	1.00	9.69	1.08
		[0.67; 1.50]	[12.12; 37.39]	[4.20; 26.71]	[0.67; 1.49]	[5.36; 17.49]	[0.60; 1.94]
Met1		1.07	15.87	6.82	0.90	8.35	1.55
		[0.69; 1.66]	[9.04; 27.88]	[2.71; 1 7.20]	[0.60; 1.35]	[4.63; 15.08]	[0.82; 2.94]
Met2		1.12	49.83	13.62	1.06	12.52	1.75
		[0.75; 1.68]	[23.38; 87.52]	[5.40; 34.34]	[0.71; 1.59]	[6.93; 22.60]	[0.97; 3.14]
Met3		1.55	37.93	15.80	1.90	8.17	1.19
		[1.02; 2.32]	[21.60; 66.61]	[6.27; 39.83]	[1.28; 2.84]	[4.53; 14.75]	[0.66; 2.13]
Met4		1.59	42.65	9.13	1.36	11.98	2.79
		[1.06; 2.39]	[24.29; 74.91]	[3.62; 23.02]	[0.91; 2.03]	[6.63; 21.62]	[1.55; 5.01]
Met5		1.31	41.79	30.24	1.80	10.12	2.38
		[0.87; 1.96]	[23.79; 73.38]	[11.99; 76.22]	[1.21; 2.69]	[5.61; 18.28]	[1.33; 4.29]

Variance source	df	*P* – value	
Treatment	6	<0.001	0.001
HPS	2	<0.001	<0.001
Linear effect	1	<0.001	0.004
Quadratic effect	1	<0.001	<0.001
HPS × Treatment	12	0.619	0.465
L-Cysteine	1	<0.001	<0.001
HPS × L-Cysteine	2	0.857	0.722
Supp. Met	5	0.018	0.120
Linear effect	1	0.002	-[2]
Quadratic effect	1	0.864	-[2]
Cubic effect	1	0.740	-[2]
HPS × Supp. Met	10	0.477	0.356

[1]Analyses were performed as $\log_2$ relative expression, but values are reported as fold-change (95% confidence interval [lower limit; upper limit]) and are least-square means initially derived from 1 bird per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 HPS). Abbreviations: LPS, lipopolysaccharide; HPS, hours post-stimulation; supp., supplemental.

[2]*P* – value was not calculated because the higher-order effect was not significant ($P > 0.05$).

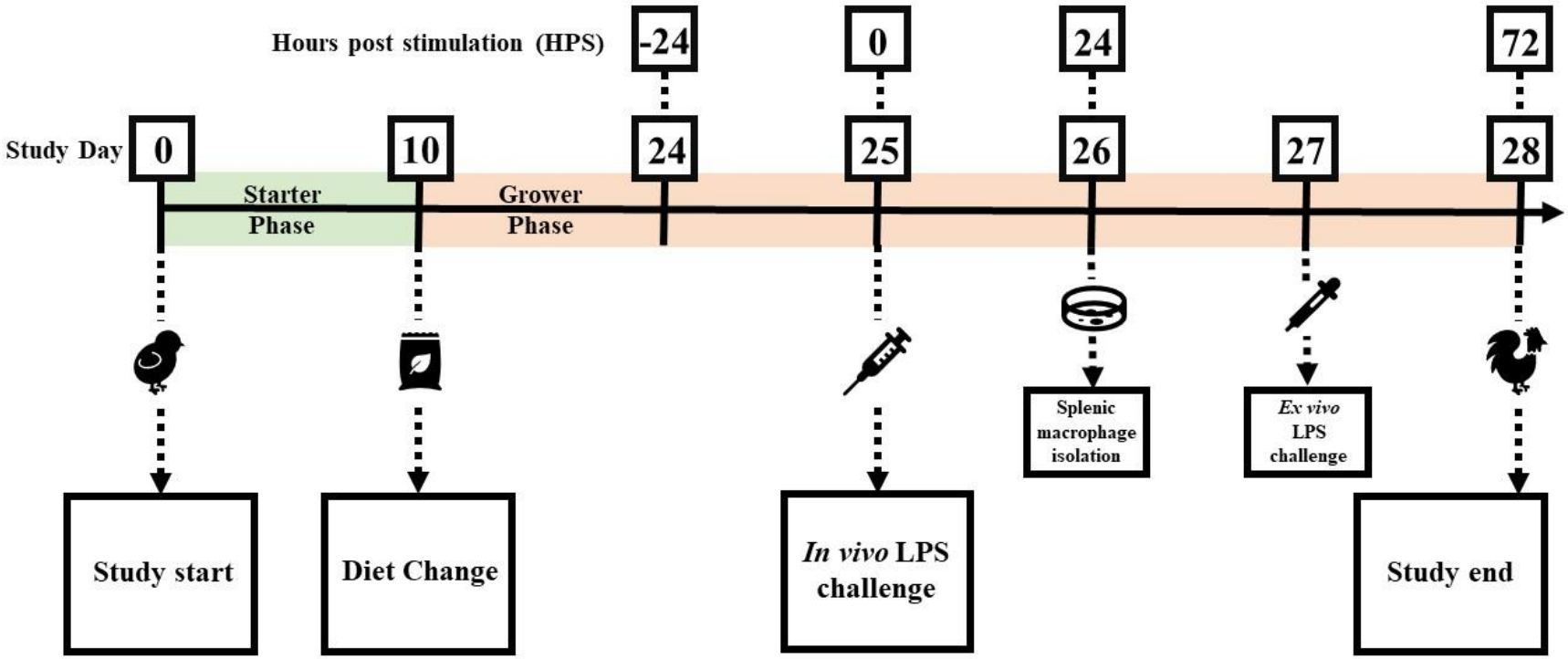

Figure 3.1. Experimental timeline.

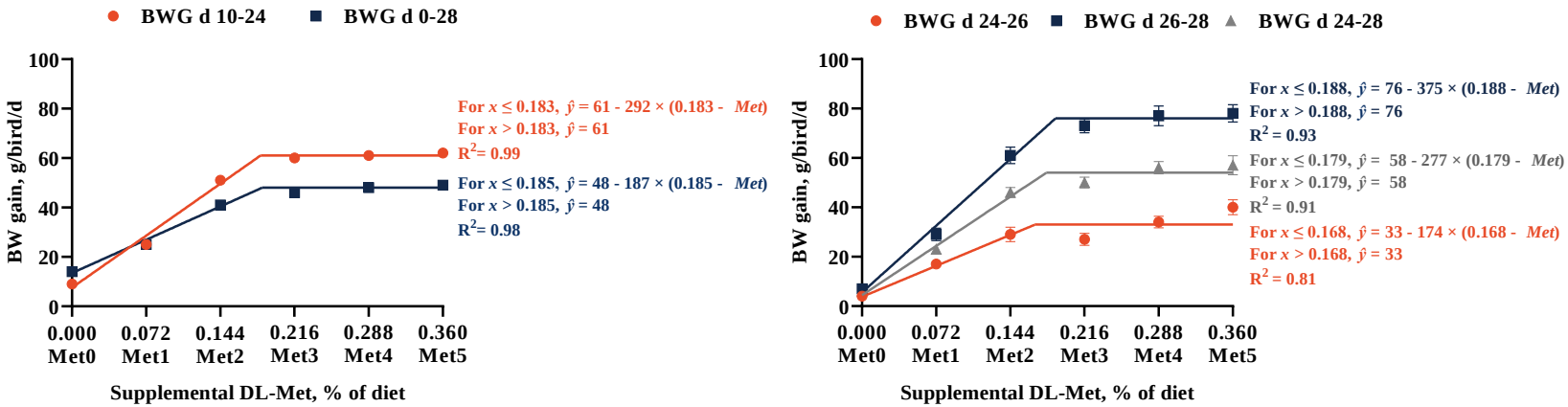

Figure 3.2. Supplemental DL-Met (% of diet) versus body weight (BW) gain (g/bird/d) of broilers fed graded levels of DL-Met during the grower phase (d 10 – 28) and challenged with lipopolysaccharide (LPS). The error bars represent the pooled standard error of the mean and are only visible if the region it encompasses was larger than the symbol. Values are least-square means initially derived from 10 birds per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of BW of LPS on d 25 (0 hours post-stimulation). The LTSAA diet was removed from the data set to model a dose-response. A 1-slope broken-line regression model was fitted to the data and used to estimate the requirement of supplemental DL-Met. The Met0 diet was calculated to contain 0.15% of total Met and 0.51% of total cysteine. For d 10 – 24, the requirement of supplemental DL-Met was estimated to be 0.18% (0.33% total Met) of the diet to achieve a maximal BW gain of 61 g/bird/d. For d 0 – 28, the requirement of supplemental DL-Met was estimated to be 0.19% (0.34% total Met) of the diet to achieve a maximal BW gain of 48 g/bird/d. For d 24 – 26, the requirement of supplemental DL-Met was estimated to be 0.17% (0.32% total Met) of the diet to achieve a maximal BW gain of 33 g/bird/d. For d 26 – 28, the requirement of supplemental DL-Met was estimated to be 0.19% (0.34% total Met) of the diet to achieve a maximal BW gain of 76 g/bird/d. For d 24 – 28, the requirement of supplemental DL-Met was estimated to be 0.18% (0.33% total Met) of the diet to achieve a maximal BW gain of 58 g/bird/d.

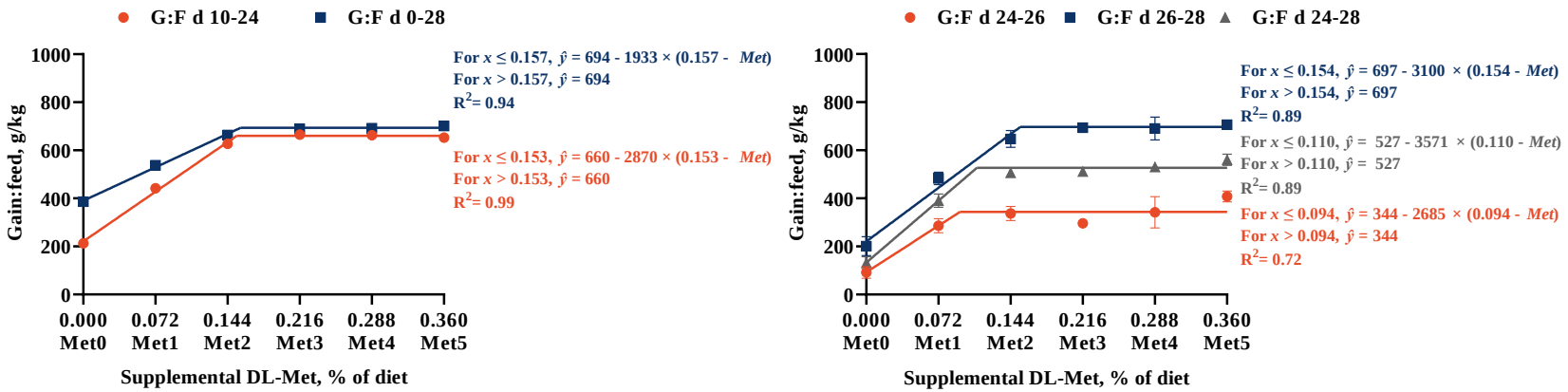

Figure 3.3. Supplemental DL-Met (% of diet) versus gain:feed (g/kg) of broilers fed graded levels of DL-Met during the grower phase (d 10 – 28) and challenged with lipopolysaccharide (LPS). The error bars represent the pooled standard error of the mean and are only visible if the region it encompasses was larger than the symbol. Values are least-square means initially derived from 10 birds per replicate cage, 7 replicate cages per dietary treatment. Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 (0 hours post-stimulation). The LTSAA diet was removed from the data set to model a dose-response. A 1-slope broken-line regression model was fitted to the data and used to estimate the requirement of supplemental DL-Met. The Met0 diet was calculated to contain 0.15% of total Met and 0.51% of total cysteine. For d 10 – 24, the requirement of supplemental DL-Met was estimated to be 0.15% (0.30% total Met) of the diet to achieve a maximal gain:feed of 694 g/kg. For d 0 – 28, the requirement of supplemental DL-Met was estimated to be 0.16% (0.31% total Met) of the diet to achieve a maximal gain:feed of 660 g/kg. For d 24 – 26, the requirement of supplemental DL-Met was estimated to be 0.09% (0.24% total Met) of the diet to achieve a maximal gain:feed of 344 g/kg. For d 26 – 28, the requirement of supplemental DL-Met was estimated to be 0.15% (0.30% total Met) of the diet to achieve a maximal gain:feed of 697 g/kg. For d 24 – 28, the requirement of supplemental DL-Met was estimated to be 0.11% (0.26% total Met) of the diet to achieve a maximal gain:feed of 527 g/kg.

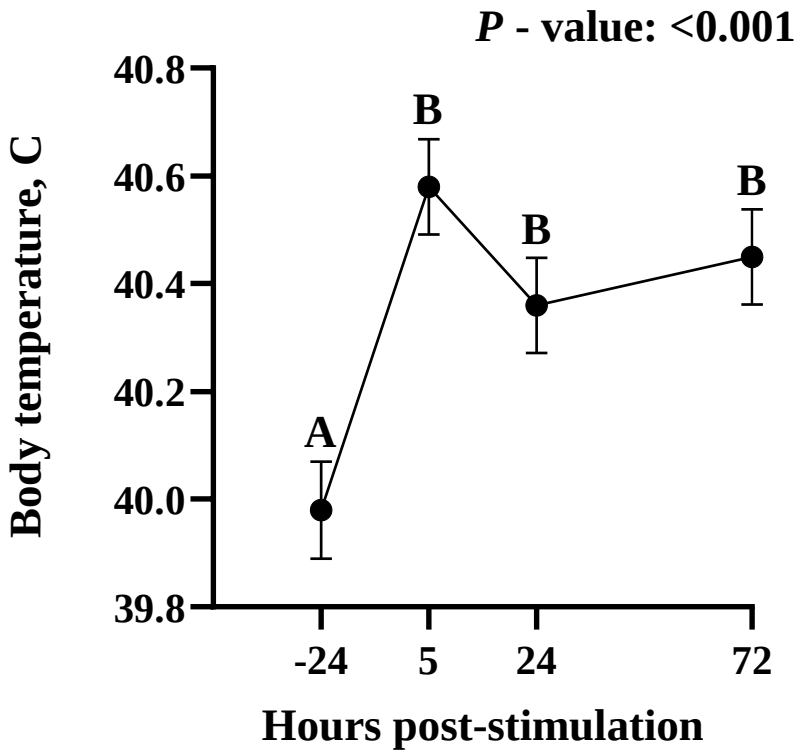

Figure 3.4. Body temperature of broilers during a lipopolysaccharide (LPS) challenge in the grower (d 10 – 28) phase. All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 [0 hours post-stimulation (HPS)]. Values are least-square means initially derived from 1 bird per replicate cage, 49 replicate cages per time-point (i.e., main effect of HPS). The error bars represent the pooled standard error of the mean.

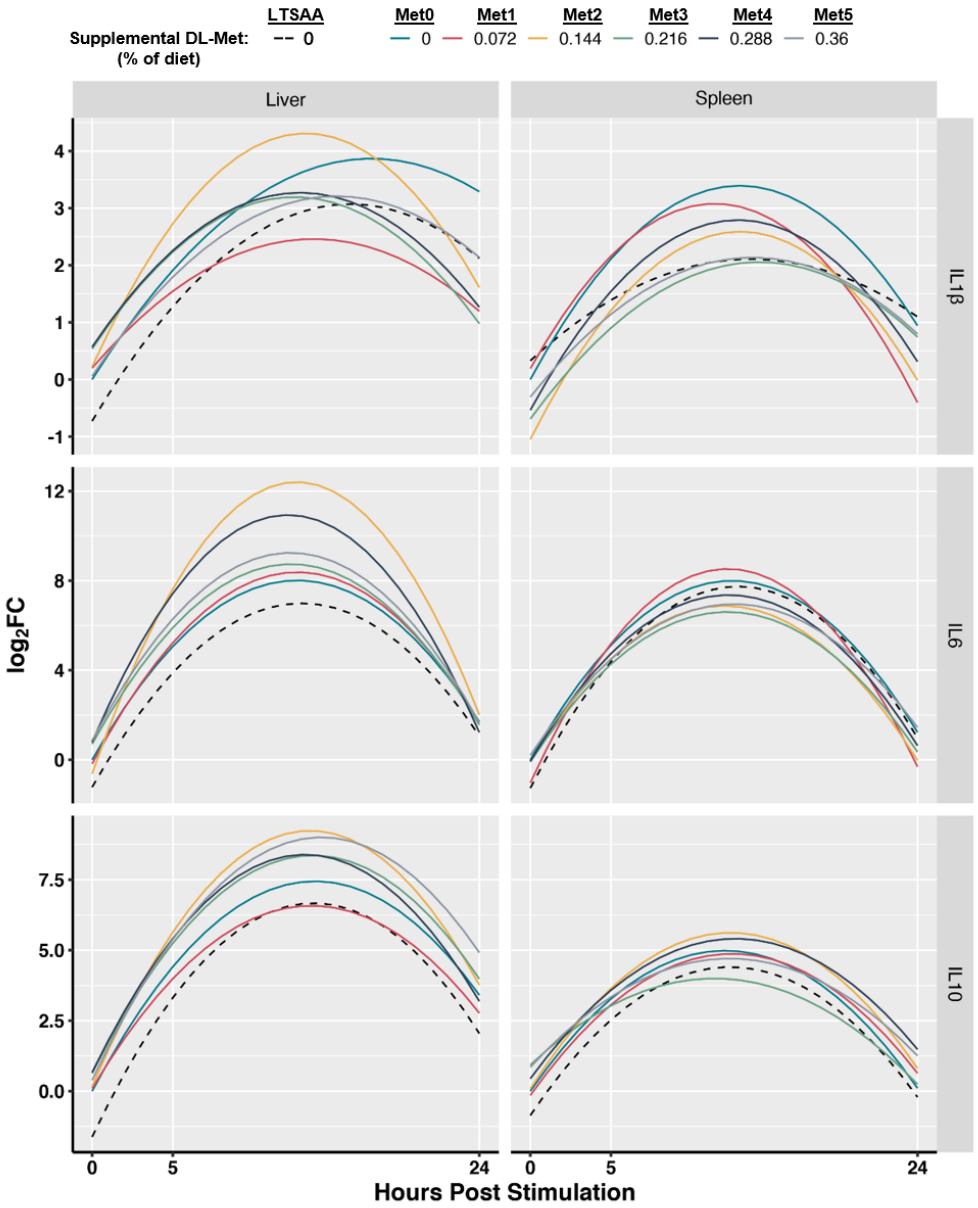

Figure 3.5. Regression plots of the $\log_2$ fold-change ($\log_2$FC) of each dietary treatment of hepatic and splenic mRNA expression of interleukin IL-1β (*IL-1β*), interleukin-6 (*IL-6*), and interleukin-10 (*IL-10*) during an *in vivo* lipopolysaccharide (LPS) challenge of broilers fed varying levels of DL-Met during the grower phase (d 10 – 28). Broilers were fed 1 of 7 low-CP dietary treatments during the grower phase (d 10 – 28): low total sulfur amino acid (LTSAA), LTSAA supplemented with 0.36% L-cysteine (Met0), and the Met0 supplemented with graded levels of DL-Met (0.072% increments; Met1 – Met5). All birds were challenged intraperitoneally with 2.0 mg/kg of body weight of LPS on d 25 [0 hours post-stimulation (HPS)]. Values are least-square means initially derived from 1 bird per replicate cage, 7 replicate cages per dietary treatment. The FC was first calculated relative to 0 HPS within an individual dietary treatment of a gene-tissue combination and then additionally baseline-corrected within a gene-tissue combination to dietary treatment Met0. All estimated regression lines are shown, regardless of whether significance was accepted. The time-point -24 HPS served as a proxy for 0 HPS.

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APPENDIX A: RESIDUAL COVARIANCE STRUCTURES

Table A1. Residual (co)variance structures of the selected models[1]

	Gene		
Tissue	*IL1β*	*IL6*	*IL10*
Liver	UN	CS	$\boldsymbol{I}_3\sigma^2_{e_k}$
Spleen	CS	$\boldsymbol{I}_3\sigma^2_{e_k}$	$\boldsymbol{I}_3\sigma^2_{e_k}$

[1]Abbreviations: UN, unstructured; CS, compound symmetry; $\boldsymbol{I}_3\sigma^2_{e_k}$, heterogeneous residual variance for each HPS.