Effects of Nutritional Manipulation on Poultry under Normal and Stressful Conditions

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Scholarly Abstract

Genetic selection for improved performance has necessitated the frequent update of amino acid requirements to support this improvement. The first objective of this dissertation was to investigate the tryptophan requirement of laying hens in peak production and the lysine and sulfur amino acid requirements of broiler chicks under a phase feeding scenario using different models. Digestible tryptophan requirements were estimated to be 137 for egg production, 133 for egg mass, and 133 for feed efficiency using the linear broken-line model. The lysine and sulfur amino acid requirement were estimated by conducting 3 experiments within the starter phase from 2 to 5, 5 to 8, and 8 to 11 days of age. The linear broken line estimate for digestible lysine was 1.22, 1.17 and 1.16% for BWG and 1.31, 1.21, and 1.14% for FCR in experiments 1, 2, and 3, respectively. The linear broken line estimate for sulfur amino acids was 0.82, 0.81 and 0.94% for BWG and 0.82, 0.80, and 0.90 for FCR in experiments 4, 5, and 6, respectively. The lysine requirements decreased from 2 to 5 to 8 to 11 days, however the sulfur amino acid requirements increased during that same period. This could be due to other roles that sulfur amino acids play in the body other than growth.

The second objective of this dissertation was to investigate the effects of a direct fed microbial (DFM) on broilers exposed to a cyclic heat stress in 2 consecutive experiments. In this experiment, the heat stress treatment reduced body weight gain and lean tissue accretion from 0 to 35 d in both experiments. In Experiment 2, when the litter was reused BWG was increased by 36 g/bird with supplementation of DFM. Ileal digestibility at 28 d (2 hr post HS) was improved with DFM supplementation in both experiments. Serum FITC-d increased with HS at both 28

and 35 d. Serum FITC-d was generally decreased with DFM at 28 d but the response was inconsistent at 35 d. Overall, the results suggest that HS reduced broiler performance and DFM treatment improved intestinal permeability and nutrient digestibility responses to HS in both experiments but did not improve performance until built up litter was used in Experiment 2.

The last objective of this dissertation was to investigate the effects of sulfur amino acids (SAA) on broilers exposed to a cyclic heat stress. As expected, HS reduced BWG and worsened FCR. The supplementation of SAA had no effect on live performance. At 28 d of age, supplementation of SAA to birds exposed to HS resulted in reduced intestinal permeability. The interaction was lost at 31 d, but HS still increased intestinal permeability ($P \leq 0.05$). Potential oxidative damage was reduced by increased SAA supplementation as indicated by an increase in the reduced glutathione to oxidized glutathione ratio. These data suggest that intestinal permeability is compromised acutely to at least three days of heat exposure before the bird can adjust, but oxidative damage is more chronic building over the entire 7 d HS period. SAA might have some protective effect on both intestinal permeability and oxidative stress responses to HS.

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General Public Abstract

To provide low-cost meat and eggs to consumers, the poultry industry must focus on improving efficiency as well as reduce the impact of stressors within the environment. This is achieved mostly by genetic selection which has resulted in significant improvements in both egg production in laying hens and body weight in broiler chickens. To support this improvement in growth rate birds would require a higher amino acid dense diet to support the increased protein output. One objective of this dissertation was to update the requirement of three key amino acids (tryptophan, lysine, and sulfur amino acids) in both laying hens and broilers for better formulation of diets. These amino acids that were investigated are essential in poultry diets and cannot be produced by the birds. The results from the tryptophan experiment indicated that laying hens need 137 mg of tryptophan per day to maintain maximum egg production. Results from the lysine and sulfur amino acid requirement experiment in broiler chickens indicated that broilers need 1.31% lysine and 0.90% sulfur amino acids to support maximal growth, but these requirements change over time and would need to be adjusted based on the broiler's age.

Heat stress in the poultry industry is a major challenge which can affect the growth rate as well as the welfare of broiler chickens. The second objective of this dissertation was to investigate different strategies to ameliorate the effects of heat stress. Supplementing direct fed microbials or probiotics in broiler diets has been proposed as one of those strategies. An experiment was conducted to investigate the effect of the supplementation of a DFM on broiler chickens exposed to heat stress. The results indicated that the DFM was able to ameliorate the negative impacts of the heat stress on nutrient digestibility and intestinal permeability but did not

improve the growth of the chickens. Another experiment was conducted to evaluate another strategy to ameliorate the effects of heat stress on broiler chickens which was the supplementation of sulfur amino acids. The sulfur amino acids are not only used for protein synthesis but have other physiological roles in the body that are important specifically during heat stress. Results from this experiment indicated that sulfur amino acids were able to ameliorate the negative effect of heat stress on intestinal permeability and oxidative stress but did not improve the performance of chickens subjected to the heat stress.

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CHAPTER 1: GENERAL INTRODUCTION

The goals of modern agriculture are to produce consumable products (usually food related) using renewable nutritional resources in an economical, humane and sustainable process. Poultry producers use domesticated avian species to provide, meat, eggs, feathers, and fertilizer among other products. Nutritional and environmental advances in technology and scientific understanding have been used effectively to improve the efficient production of poultry. The objectives of this dissertation will move forward the scientific understanding of both nutrition and environmental management of various poultry species to continue to drive the efficient production of poultry meat and eggs in the United States and world-wide.

Genetic selection has resulted in an annual improvement in egg production (2 to 3 eggs) and feed efficiency (FE) (1 to 2 point reduction) of laying hens from 2010 to 2015 (Preisinger, 2018). This continual improvement in productivity necessitates the reevaluation of amino acid requirements to support this genetic potential. In commercial laying hen nutrition, the requirements for the first three limiting amino acids (Methionine or Sulfur amino acids (SAA), Lysine (Lys), and Threonine (Thr) are well studied as synthetic amino acids have been available commercially. The fourth limiting amino acid on the list is Tryptophan (Trp). The recent commercial production and use of crystalline L-Trp has allowed nutritionists to further reduce dietary crude protein and formulate cost-effective diets that still meet the nutritional requirements of laying hens during the production cycle. Tryptophan is important in protein synthesis and egg production, but also is a precursor in the serotonin and kynerunine pathways. Limited data are available on the Trp requirement in laying hens from 30 to 80 weeks of age and most of the existing publications are over 10 years old (Russell and Harms, 1999; Harms and Russell, 2000; Wen et al., 2019). Additionally, none of the previous experiments have

investigated the requirement of Trp as the pullets are transitioning into egg production. Therefore, the first objective of this dissertation was to determine the Trp requirements of caged white egg laying hens from 22 to 34 weeks of age (Chapter 3).

Broiler amino acid requirements unlike laying hen requirements have been well researched within the literature. The first and second limiting amino acids in broilers Lys and SAA have well established requirement and updated with growth changes in broilers. However, the requirements have typically been estimated over an entire phase feeding period. The studies have relied on an assumption that requirements will decrease as feed intake increases with age (Emmert and Baker, 1997; Pope et al., 2002). In 1997, Emmert and Baker introduced the concept of using regression equations to predict short term requirements which would smooth out the large drops in amino acid requirements in the transition between starter to grower to finisher diets. This would also reduce the cost of diets by reducing cost of added protein and synthetic amino acids. This can potentially be achieved by blending diets from two different feed bins within a phase to better meet the requirements of the birds (Pope et al., 2002). Therefore, an experiment was conducted to validate the assumption that dietary amino acid requirements would decrease over time as birds age within a feeding phase. Lysine and SAA are the limiting amino acids in common poultry diets and are crucial for growth and muscle development (Garcia and Batal, 2005). Sulfur amino acids are also crucial for feather production and can serve as a methyl donor in several important processes within the body (Kalinowski et al., 2003). The second objective of this dissertation was to determine the requirements of Lys and SAA of broiler chickens from 3 to 6, 6 to 9 and 9 to 12 d of age when fed a starter diet (Chapter 4).

Broiler production in the United States is primarily located in the Southeast and Midwest, both areas where summer temperatures can cause bouts of heat stress (HS). Although dated at

this point, a 2003 economic analysis found that heat stress accounted for $128 million in mortality and production losses to the U.S. poultry industry per year (St. Pierre et al., 2003). It can be expected that this value is currently underestimated due to increased animal growth rates, increased total poultry numbers, and inflation. Physiologically, HS causes decreased activity and reduced feed intake as to reduce heat production associated with movement and feed digestion (Mack et al., 2013). Additionally, poultry will change their posture, lifting wings and stretching out to expose areas of the body with low feather cover to increase the temperature loss from the body (Mack et al., 2013). Additionally, broilers will pant under higher temperatures using evaporative cooling to increase heat loss. Although panting increases the evaporation of water from the lungs thus reducing body temperature, it also increases the amount of CO_2 lost from the circulatory system which may result in an acid base imbalance (Teeter et al., 1985). Partially due to the changes in physiology and behavior associated with HS, elevated environmental temperatures result in reduced body weight, poor feed efficiency and increased mortality within a flock, ultimately leading to lower profits for the producer and a welfare concern for the animals.

Although amino acids are primarily used to support maintenance, growth, and production, as noted above, they have secondary effects on metabolism. Sulfur amino acids are linked to increased antioxidant function to counteract reactive species in poultry exposed to HS (Willemson et al., 2011; Del Vesco et al., 2015). Heat stress overtime or chronic HS can result in oxidative damage to functional organs and tissues within the body resulting from an increase in reactive oxygen species (ROS) causing cellular injury and ultimately negatively affecting organ function (Del Vesco et al., 2015). Supplemental SAA can be metabolized to glutathione which is an important antioxidant used by poultry especially when broilers are subjected to HS. Another objective of this dissertation was to investigate the effects of supplementation with SAA to

broiler chickens over the entire growing period which were subjected to cyclic heat stress from 28 to 35 d of age (Chapter 5).

Although alterations in the diet can have an effect on broiler responses to HS, bird environment can also play a role in the response of broilers to high environmental temperatures. The US poultry industry reuses litter, with composting and rest between flocks, as a method to raise broilers more efficiently by minimizing excess bedding material use and providing a more consistent substrate for litter microbiota (Wiedemann, 2015). Direct fed microbials (DFM) are one of the current antibiotic alternatives for poultry. The DFMs function by modulating the microflora in the host favoring a more balanced intestinal environment (Fuller, 1989). Previous studies have demonstrated that DFMs can alter the microbial community within the litter by competitively excluding the gram-negative bacteria from the environment (Pedroso et al., 2013; Li et al., 2014). Additionally, DFMs can improve the digestibility of nutrients and increase surface area for absorption within the intestine through the secretion of beneficial enzymes such as proteases required for digestion which is important during HS when the energy demand is high (Forte et al., 2016). Therefore, an objective of the dissertation was to determine the effect of DFM supplementation on broilers exposed to a cyclic heat stress raised on built up litter over two consecutive flocks (Chapter 6).

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CHAPTER 2: LITERATURE REVIEW

2.1 AMINO ACIDS

2.1.1 INTRODUCTION

Proteins are made up of building blocks called amino acids. These 22 amino acids comprise all proteins. Amino acids are classified in three different groups: essential, non-essential, and conditionally essential. Essential amino acids, or indispensable amino acids, are those that cannot be synthesized by the body and thus need to be supplemented in the feed. On the other hand, non-essential amino acids can be synthesized inside the body and no supplementation is needed. Conditionally essential amino acids are not required in normal conditions but can be essential in times of disease and stress. The essential amino acids in poultry are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. However, in practical poultry diets arginine, Lys, SAA, Thr, and Trp are the amino acids that are most critical in diet formulation (Emmert and Baker, 1997).

2.1.2 PROTEIN AND AMINO ACID DIGESTION AND ABSORPTION IN POULTRY

Dietary protein must be broken down into free amino acids, dipeptides, or tripeptides in the gastrointestinal tract of poultry prior to its absorption by enterocytes (Webb et al., 1992). The digestion in poultry starts in the proventriculus and gizzard where it is hydrolyzed using pepsin and hydrochloric acid. Following gastric digestion, dietary protein is digested by pancreatic proteases such as trypsin, chymotrypsin, and elastase in the small intestine. Some of these proteases are specific to single amino acids and others can release multiple amino acids (Folk et al., 1960). The final digestion occurs at the brush border membrane of the small intestine using several peptidases such as endopeptidases, carboxypeptidases and aminopeptidases which reduce

oligopeptides into free amino acids, dipeptides, and tripeptides. Additional dietary protein can also be fermented by the microbiota in the ceca of birds which can produce volatile fatty acids. Peptides shorter than four amino acids can be transported into the epithelium by cotransport with H^+ ions using a transporter known as PepT1. Free amino acids have four sodium dependent amino acid transporters that are specific to acidic, basic, and neutral amino acids. The transporter binds to the sodium and to the amino acid. Then the transporter transfers them both into the cytoplasm. Once in the epithelial cells, peptides can be degraded by peptidases in the cytoplasm to create free amino acids. The amino acids and a small number of small peptidases are transferred from the cytoplasm into circulation using additional transporters.

2.1.3 TRYPTOPHAN

2.1.3.1 INTRODUCTION

Tryptophan is one of the essential amino acids that is required in poultry diets as it is not synthesized by animals. Tryptophan is the fourth limiting amino acid in common laying hen diets (Peganova and Eder, 2003). The active form of Trp in poultry is L-Trp. Tryptophan is found mostly in soybean and poultry byproduct meal with lower levels in corn gluten meal and corn. A feed grade L-Trp has recently been commercialized which allows for more accurate formulation of amino acids to meet the requirement for maximizing growth or egg production.

2.1.3.2 TRYPTOPHAN FUNCTIONS

Tryptophan is required for protein synthesis as well as to support egg production of laying hens (Harms and Russell, 2000). Additionally, Trp is involved in several metabolic processes within the body. Tryptophan is the precursor of serotonin which is an important neurotransmitter involved with stress response, sleep, and appetite control (Gibbons et al., 1979;

Adeola and Ball, 1992). A previous experiment demonstrated that 2% Trp in the diet was able to reduce gentle feather pecking and plasma corticosterone after 5 min of restraint in broiler chickens compared to broilers fed 0.16% (Van Hierden et al., 2004). Tryptophan can be converted to niacin which is an essential vitamin in the body through the kynurenine pathway. Baker and coworkers (1973) investigated the effects of supplementation of Trp to a basal diet with no niacin compared to a diet with niacin with Trp fed to requirement. They reported that birds supplemented with additional Trp were able to maintain the same body weight gain (BWG) and feed conversion ratio (FCR). They concluded that over 2% of Trp not used for protein production is converted to niacin in broiler chicks.

2.1.3.3 TRYPTOPHAN REQUIREMENT OF LAYING HENS

The Trp requirement for laying hens according to the national research council (NRC) (1994) is 160 mg/day. The requirement for Trp according to the Hy-Line W-36 management guide is 155 to 168 mg/d based on the production phase (Hy-Line, 2018). Wethli and Morris (1978) found that the requirement of Trp was 184 mg/d from 32 to 40 weeks of age, 174 mg/d from 63 to 73 weeks of age, and 179 mg/d from 97 to 106 weeks of age after molting for egg production using a quadratic polynomial model. This indicated that the requirement is reduced with higher feed intake from 63 to 73 weeks of age compared to 32 to 40 weeks of age. This also indicated that the requirements after molting from 97 to 106 weeks also increase as the laying hens are coming back into production. Another experiment conducted with white leghorns at 38 to 50 and 30 to 85 weeks estimated a requirement of 123 mg/d and 95 mg/d, respectively for egg production using a linear broken line model (Jensen et al., 1990). However, in this experiment the laying hens had low feed intake which resulted in lower egg production which implies lower requirements. Russell and Harms (1999) found a requirement of 136 mg/d of Trp from 53 to 59

weeks of age in Hy-line W-36 laying hens for egg production using a linear broken line model. A follow-up study on laying hens from 28 to 36 weeks of age reported a requirement of 149 mg/day for maximum egg production (Harms and Russell, 2000). This indicated that the highest requirements are at peak when laying hens are at maximum egg production and reduce as the birds are older with increased feed intake. Cardoso and coworkers (2014) reported a requirement for 212 mg/d for egg production in Dekalb white layers from 60 to 76 weeks of age using a quadratic polynomial model. A more recent experiment conducted with Hy-Line W-36 laying hens from 41 to 60 weeks of age found a requirement of 153 mg/d for egg production using the linear broken line model (Wen et al., 2019). This experiment was conducted 20 years after the Russell and Harms (1999) and resulted in a 17 mg/d increase in Trp requirement in the same breed of laying hens at similar age. This implies that the Trp requirement has indeed increased with improved genetic selection.

Table 1. Summary of Trp requirements in laying hens from the literature

Citation	Trp requirement (mg/d)	Age (Wks)	Model used	Strain
NRC, 1994	160	All	N/A	All strains
Hy-Line W-36, 2018	155/168	All	N/A	Hy-Line W-36
Wethli and Morris, 1978	184	32-40	Quadratic broken line	White Leghorn
Wethli and Morris, 1978	174	63-73	Quadratic broken line	White Leghorn
Wethli and Morris, 1978	179	97-106	Quadratic broken line	White Leghorn
Jensen et al., 1990	123	38-50	Linear broken line	White Leghorn
Jensen et al., 1990	95	30-85	Linear broken line	White Leghorn
Russell and Harms, 1999	136	53-59	Linear broken line	Hy-Line W-36
Harms and Russell, 2000	149	28-36	Linear broken line	Hy-Line W-36
Cardoso et al, 2014	212	60-76	Quadratic polynomial	Dekalb White
Wen et al., 2019	153	41-60	Linear broken line	Hy-Line W-36

2.1.4 SULFUR AMINO ACIDS

2.1.4.1 INTRODUCTION

The sulfur amino acids are methionine and cysteine and are considered to be first limiting in corn-soybean meal-based diets. Methionine can be converted to cysteine in the body through the transulfuration pathway thus we can look at their requirement as a combination of methionine + cysteine (Graber and Baker, 1971). Sulfur amino acid requirements can vary based on age, sex, strain, and stress level (Lumpkins et al., 2007).

2.1.4.2 SULFUR AMINO ACID FUNCTIONS

Sulfur amino acids are important for growth and muscle development like all other amino acids (Garcia and Batal, 2005). They are also a major component of feathers and are critical for feather formation in poultry species (Kalinowski et al., 2003). However, SAA have other roles that are important for the healthy function of the body. SAA are precursors for S-adenosyl methionine which is an important methyl donor for several metabolic reactions and DNA methylation (Niculescu et al., 2002). Additionally, SAA are important for cell differentiation and development (Shiraki et al., 2014). SAA deficiency leads to reduced growth and feed efficiency as well a reduced egg production in laying hens (Garcia and Batal, 2005). A deficiency of SAA also results in poor feather growth and increased feather pecking to satisfy the requirement.

2.1.4.3 SULFUR AMINO ACID REQUIREMENT IN BROILERS

The total SAA requirements for broilers were determined to be 0.90%, 0.72%, and 0.60% for 0 to 3, 3 to 6, and 6 to 8 weeks of age (NRC, 1994) (Table 2). Baker and Han (1994) found the requirement of digestible SAA during the first 3 weeks to be 0.81% and 0.75% for males and

females, respectively. Schutte and Pack (1995) estimated the digestible SAA for growing broilers from 14 to 34 days of age to be 0.75% based on FCR and breast meat yield. Another experiment determined the SAA using 6 graded levels and found a requirement of 0.58% and 0.60% using the linear broken line model for weight gain and feed efficiency, respectively (Baker et al., 1996).

Garcia and Batal (2005) determined a requirement of 0.82% and 0.84% digestible SAA for BWG and FCR from 0 to 7 days of age and 0.79% and 0.82% at 21 days of age for BWG and FCR in 2 experiments using a linear broken line method. Lumpkins and coworkers (2007) investigated the difference in SAA requirement between males and females from 0 to 3 weeks of age and found a requirement of 0.75% and 0.76% for BWG and FCR in males, however in females the requirement was higher for FCR than BWG (0.79% vs. 0.75%). In the same study, they determined that broilers raised in floor pens had a higher requirement for FCR but the same requirement for BWG compared to broilers raised in battery cages (0.68% vs 0.63%). A more recent experiment investigating the SAA requirement using L-methionine instead of DL-methionine found a requirement of 0.69% for BWG from 0 to 10 days, 0.66% and 0.62% for BWG and FCR from 11 to 23 days, and 0.56% and 0.62% for BWG and FCR from 24 to 35 days of age in Ross 308 male broilers using the linear broken line method (Millecam et al., 2021). Comparing these results, there is no pattern for increase or decrease in SAA requirement in the literature. The results also indicate that the requirement for SAA is affected by age, sex, genetic line and could vary based on source of methionine and environment.

Table 2. Summary of SAA requirements in broiler chickens from the literature

Citation	SAA requirement BWG		Age	Strain
	Male	Female		
	(%)		(d)	
NRC, 1994	0.90	0.90	0-21	All
NRC, 1994	0.72	0.72	21-42	All
NRC, 1994	0.60	0.60	42-56	All
Baker and Han, 1994	0.81	0.75	0-21	Ross 308
Schutte and Pack, 1995	0.75	N/A	14-34	N/A
Baker et al., 1996	0.58	N/A	0-21	Ross 308
Garcia and Batal, 2005	0.82	N/A	0-7	Cobb 500
Garcia and Batal, 2005	0.79	N/A	0-21	Cobb 500
Lumpkins et al., 2007	0.75	0.79	0-21	Cobb 500
Millecam et al., 2021*	0.69	N/A	0-10	Ross 308
Millecam et al., 2021*	0.56	N/A	11-23	Ross 308

* L-methionine supplemented instead of DL-methionine

2.1.4.4 SULFUR AMINO ACID ROLE TO COUNTERACT REACTIVE OXYGEN SPECIES

Reactive oxygen species can have negative effects on the body which can result in oxidative stress. The ROS are produced in all cells within the body that contain a mitochondrion (Lambert and Brand, 2009). Under normal conditions, the ROS is balanced with several endogenously produced antioxidants within the body such as superoxide dismutase and catalase (Figure 1). The body utilizes several antioxidant systems to counteract the effect of these free radicals and one of the most important antioxidants within the body is glutathione. Glutathione is synthesized from glutamate, cysteine and glycine within the body through 2 reactions that are ATP-dependent. Glutathione plays a role in scavenging oxidative radicals. Oxidative radicals or ROS are generated endogenously within the body as a byproduct of the normal metabolism of nutrients in the mitochondria. However, under more stressful conditions the increase in energy demand within the body to combat stress and return to homeostasis can result in the increase of ROS within the body (Bruskov et al. 2002). Additionally, ROS can be produced from oxidation of proteins and lipids during heat stress which can produce more radicals that can further damage cells. If antioxidants are not adequate to counteract their effects, ROS can result in DNA damage and cell death and lipid/protein oxidation. Willemsen and coworkers (2011) reported that supplementing methionine to poultry subjected to HS was able to improve the redox status in broiler chickens as indicated by an improved reduced glutathione to oxidized glutathione ratio (rGSH:GSSG) within the liver. A more recent experiment reported an improved redox status in the thigh muscle when supplementing 2 levels of SAA to broiler chickens subjected to HS (Zietz et al., 2020). This indicates that oxidative stress not only has an effect on poultry health but could also affect the meat quality of broilers after processing.

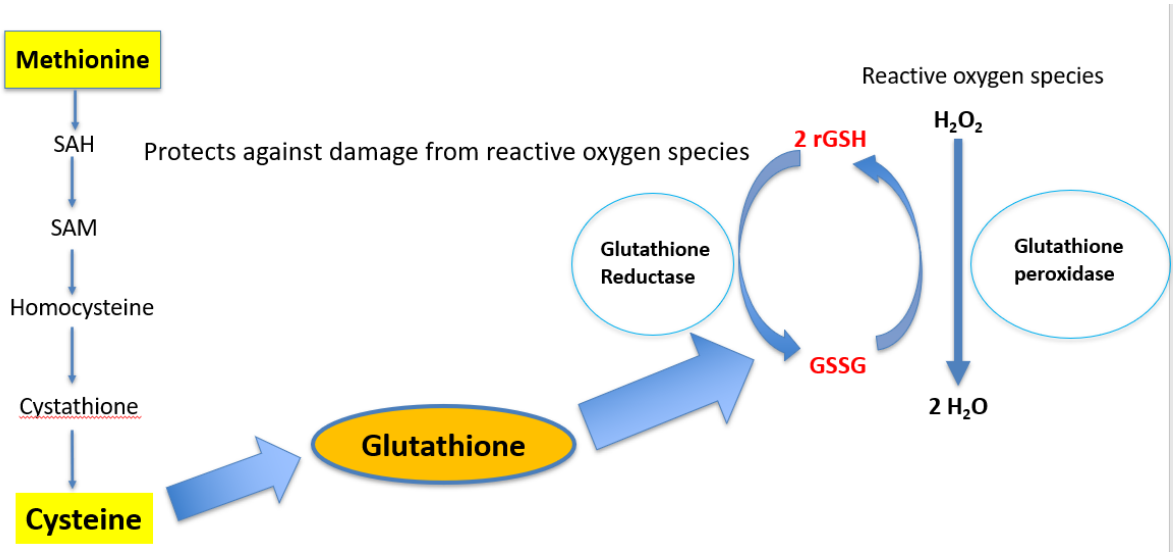

Figure 1. Conversion of methionine to the antioxidant glutathione and glutathione recycling for the reduction of reactive oxygen species

2.1.5 LYSINE

2.1.5.1 INTRODUCTION

Lysine is the second-limiting amino acid in corn-soybean meal-based diets (Garcia and Batal, 2005). Even though SAA are the most limiting in common poultry diets, Lys is used as the reference amino acid in the ideal protein concept. The ideal protein concept is the idea that the proteins within the diets need to prove an accurate balance of all amino acids for maximizing performance and protein without deficiencies of excess (Emmert and Baker, 1997). Lysine is used as the reference because it is utilized only for protein accretion and maintenance in broiler chicken where SAA have several other biological functions within the body.

2.1.5.2 LYSINE REQUIREMENT IN BROILERS

Lysine requirements depend on various factors such as age, sex, strain, and level of metabolizable energy (Table 3). According to the NRC (1994), the requirement for total Lys is 1.10%, 1.00%, and 0.85% for 0 to 3, 3 to 6, and 6 to 8 weeks of age in broiler chickens, respectively. Comparing the Lys requirement for fast vs. slow growing broilers, an early study estimated the requirement at 0.93% and 0.96% for the fast and slow growing strains for BWG from 8 to 21 days of age, respectively (Han and Baker, 1991). The requirement for FCR was not different among both strains and estimated at 0.99%. Vazquez and Pesti (1997) estimated the Lys requirement of broilers using previous data sets from broilers from the ages of 0 to 21 days of age. Their requirement estimates were 1.04% and 1.21% for BWG utilizing the linear and quadratic broken line, respectively. The requirement estimates for FCR were 1.10% and 1.32% for linear and quadratic broken line, respectively. In this experiment they concluded that the requirement for FCR might be higher than BWG during the first 21 days of age.

Labadan and coworkers (2001) estimated the total Lys requirement at 1.28%, 1.13%, 0.99%, and 0.81% for BWG at 0 to 2, 2 to 4, 3 to 6, and 5 to 8 weeks, respectively. Requirement for FCR was estimated at 1.21% and 1.00% from 0 to 2 and 3 to 6 weeks of age, respectively. The higher requirement was estimated for breast weight at 1.32%, 1.21%, 0.99%, and 0.81% for 0 to 2, 2 to 4, 3 to 6, and 5 to 8 weeks, respectively. Garcia and Batal (2005) investigated the digestible Lys requirements in male Cobb 500 broilers from 0 to 21 days of age and reported a requirement of 1.00% and 1.10% for BWG and FCR, respectively. Dozier and Payne (2012) compared the digestible Lys requirement in two different strains of female broilers from 0 to 14 days of age which they found to be 1.27% for BWG in Ross x Ross 708 broilers using a quadratic broken line model. Hubbard x Cobb 500 broilers had a lower requirement for BWG estimated at 1.18%. These results indicate that strain differences exist when looking at Lys requirements in broilers. A more recent experiment estimated the requirements of digestible Lys for male Cobb 500 broilers at 1.09% and 1.08% from 0 to 12 days of age using the linear broken line model for BWG and FCR, respectively (Cemin et al., 2017). These studies indicate that there is variation in lysine requirements due to genetic selection, age, and sex of the birds. Additionally, the estimates using different models could be variable during comparisons.

Table 3. Summary of Lys requirements in broiler chickens from the literature

Citation	Lys requirement BWG		Age	Strain
	Male	Female		
	(%)		(d)	
NRC, 1994	1.10	1.10	0-21	All
NRC, 1994	1.00	1.00	21-42	All
NRC, 1994	0.85	0.85	42-56	All
Han and Baker, 1991	0.93	N/A	8-21	Ross 308
Vazquez and Pesti, 1997	1.04	N/A	0-21	Ross 308
Labadan et al., 2001	1.28	N/A	0-14	Ross
Labadan et al., 2001	1.13	N/A	14-28	Ross
Labadan et al., 2001	0.99	N/A	21-42	Ross
Labadan et al., 2001	0.81	N/A	35-56	Ross
Garcia and Batal, 2005	1.00	N/A	0-21	Cobb 500
Dozier and Payne, 2012	N/A	1.27	0-14	Ross 708
Dozier and Payne, 2012	N/A	1.18	0-14	Cobb 500
Cemin et al., 2017	1.09	N/A	0-12	Cobb 500

2.1.5.3 PHASE FEEDING IN BROILER PRODUCTION

The amino acid requirement decreases steadily to satisfy growth and efficiency as the feed intake increases during the growth period. In general, the poultry industry splits feeding phases based on the specific growth period that ranges between 10 and 21 days (Dozier and Payne, 2013). Phase feeding is used as a tool to reduce the overfeeding of nutrients by shortening the growth periods of 10 to 21 days into multiple shorter periods (Warren and Emmert, 2000). Emmert and Baker (1997) demonstrated this by developing regression equations using the NRC (1994) and the Illinois ideal protein concept to predict the amino acid requirements that were reduced every 2 to 3 days of age. In an experiment conducted to validate the regression equations by comparing it to NRC (1994) amino acid recommendations, Warren and Emmert (2000) found that broilers had similar BWG and FCR from 0 to 21 and 40 to 61 days of age in 2 experiments. They also concluded that there is a significant cost savings in using the phase feeding regimen over the NRC requirement.

Pope and Emmert (2001) continued this work by comparing the NRC requirements to a phase feeding regimen fed at 90% and 100% of amino acid requirements based on the regression equations where the first three limiting amino acids were reduced weekly. In this study, they reported that there was no difference between the NRC requirement and phase feeding on BWG and FCR from 43 to 64 days of age and further reducing the amino acids by 10% did not have any negative effects. A follow up study was conducted using the same experimental design, but diets were changed every other day by mixing high and low nutrient dense diets together to satisfy the requirements according to the regression equation (Pope et al., 2002). The authors concluded that there were no negative impacts on any of the performance parameters from 42 to 63 days of age when using phase feeding compared to the NRC recommendations. Additionally, a reduction in lysine, SAA, and threonine intake was observed with all phase feeding treatments.

A more recent study evaluated a changing feed every other day by mixing a high and low nutrient diet vs. standard industry feeding in 4 different strains of commercial broilers from 17 to 58 days of age (Brewer et al., 2012). Overall, they observed that there was no difference between feeding an industry diet vs. phase feed, but phase feeding reduced feed cost 1 to 4 cents per kg of gain depending on the strain.

2.2 HEAT STRESS

2.2.1 INTRODUCTION

Elevated environmental temperature has significant impact on commercial poultry production both from a live performance and animal welfare perspective and impacts the profitability of poultry producers worldwide. An economic analysis conducted in 2003 indicated that performance and mortality losses in the United States totaled 128 million dollars per year (St. Pierre et al., 2003). Genetic selection and higher growth rates within the poultry industry since this analysis was conducted would indicate that this estimate would be increased in current production systems making HS one of the greatest stressors that affect poultry. Therefore, understanding the impacts of HS on poultry production as well as the mechanisms that affect production losses become crucial in finding strategies to counteract the negative consequences associated with elevated temperatures.

2.2.1.1 HEAT LOSS MECHANISMS IN BROILERS

Broilers lack sweat glands which help in the dissipation of heat during the heat stress period. Additionally, birds have feathers which can help regulate body temperature and insulate the birds as you approach colder ambient temperature but can inhibit heat loss when they approach the upper critical temperature (UCT) (Bharat et al., 2013) (Figure 1). There are four main

mechanisms for heat loss in all species which are radiation, conduction, convection, and evaporation. Radiation, conduction, and convection are the three sensible heat loss mechanisms which are used to maintain a constant body temperature in the thermoneutral zone (TNZ). Radiation is defined as the transfer of body heat to the air through electromagnetic waves which can be transferred to any objects within the environment. This mechanism is only effective and efficient when the ambient temperature is much lower than the body temperature of broilers. Conduction is also an important heat loss mechanism which involves dissipating heat to a cooler surface or object by having direct contact with this object. In broilers this is achieved through several ways which include having contact with walls or other cool surfaces and digging in the litter to find cooler spots or concrete. If the contact surface is cooler than the broiler body temperature, then some amount of heat can be lost through this mechanism.

Convection is the process of losing body heat to surrounding air which can be achieved by increasing the air flow within a barn. In broilers, most of this heat is lost through the comb, wattles, and wings (Guo et al., 2012). Additionally, broilers will increase the heat loss through this mechanism by increasing the surface area of contact with the air especially by spreading their wings and fluffing them to increase contact with the skin. The last mechanism of heat loss which is part of the latent heat loss mechanisms is the evaporative heat loss. Evaporative heat loss becomes crucial when the environmental temperature exceeds the UCT and the sensible heat loss mechanisms become nonfunctional. In mammals that have sweat glands this is achieved by the evaporation of the sweat and the conversion of liquid to gas which cools the body. Due to the lack of sweat glands, broilers use the panting mechanism by increasing respiration rate and using the increased heat to vaporize the water within the body and releasing the water vapor through the throat (Koelkebeck and Odom, 1994). However, long periods of panting can result in

reduction in the partial pressure of CO_2 in the blood which results in a lower amount of bicarbonate production increasing the pH causing respiratory alkalosis (Barrett et al., 2018). Respiratory alkalosis can have an impact on the performance and health of broilers subjected to HS resulting in performance and mortality losses (Koelkebeck and Odom, 1994).

2.2.1.2 HEAT STRESS LEADS TO OXIDATIVE STRESS

One of the mechanisms of HS that can have a negative effect on performance and health of the birds is an increase in reactive oxygen species (Bruskov et al., 2002). Reactive oxygen species are compounds that are generated during normal biological processes such as metabolism and are critical for several of the processes within the body such as immune function (Valko et al., 2006). Reactive oxygen species are maintained in balance in the body with various antioxidant that are produced within the body of the chicken. If the presence of ROS is in excess of the available antioxidant capacity in cells this results in oxidative stress (Figure 2). An increase in ROS can be observed when there are environment stressors as well as disease which can cause damage to lipids, proteins, and DNA (Fang et al., 2002). Yang and coauthors (2010) reported an increase in ROS when subjecting broilers to a temperature of 35 °C for 12 hours in 6-week-old broilers compared to broilers under TN temperatures. One of the explanations for this increase in ROS is the increase in digestion and absorption due to the higher demand for energy production to combat the heat stress causing a higher production of ROS. Elevated temperatures can also cause the disruption of the complexes of the electron transport chain causing an increase in ROS (Ando et. al., 1997). Oxidation of lipids from ROS can result in an increase of lipid radicals which can result in further lipid oxidation (Benzie et al., 1996). Furthermore, elevated temperatures can cause an increase in ROS production through ischemia and hypoxia. This can

cause an overload of ROS which can damage organs and tissues within the body resulting in possible health and mortality concerns.

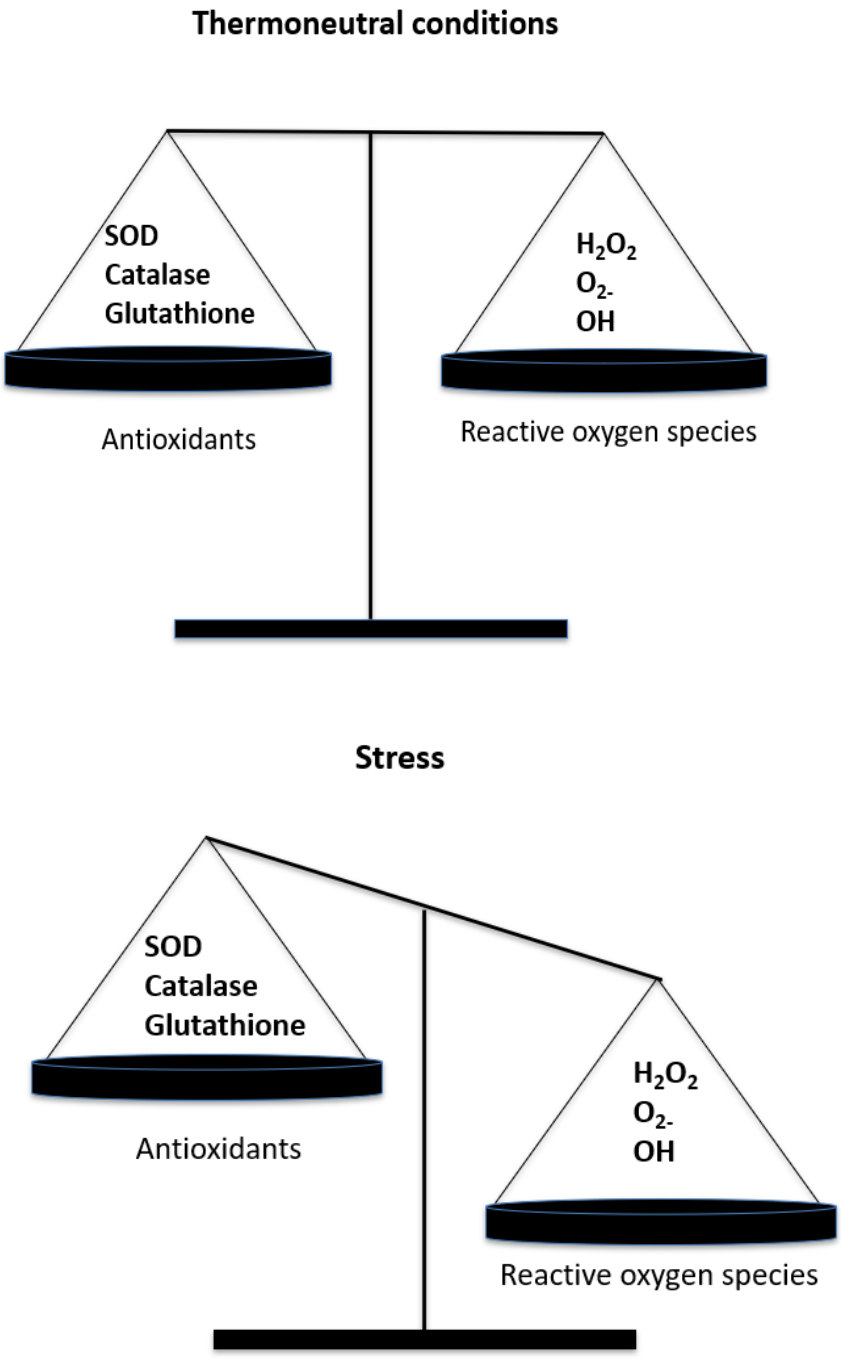

Figure 2. Antioxidant system in poultry during normal vs. oxidative stress conditions (SOD = superoxide dismutase, H_2O_2 = hydrogen peroxide, OH = hydroxyl, O_{2-} = superoxide)

2.2.1.3 EFFECTS OF HEAT STRESS ON PERFORMANCE

Modern broilers have been selected for high growth rate and improved feed efficiency. This high growth rate is largely associated with higher metabolic rate which results in greater metabolic heat production having detrimental effects on broiler chicken performance when they are subjected to elevated temperatures (De Souza et al., 2016). The effects of HS on performance are consistent within the literature (Figure 3). A meta-analysis of previous HS experiments indicated that elevated temperatures have been shown to result in a reduction in BWG, feed intake, feed efficiency, and mortality (Liu et al., 2020). However, responses vary when it comes to the difference between cyclic and continuous HS. Sohail et al. (2012) observed a 33% reduction in BWG and 16% reduction in feed intake resulting in a 25% worsening in feed efficiency from 0 to 42 days of age in broiler chickens subjected to continuous HS at 35°C from 0 to 42 days of age.

In another study, Ruff et al. (2020) concluded that broilers exposed to continuous HS at 35°C had a 42% reduction in BWG and a 54% reduction in feed intake which resulted in a 31% reduction in FCR compared to Thermoneutral (TN) group. On the other hand, broilers subject to a cyclic HS at 35°C for 8 hours per day and returned to TN temperature for the remaining 16 hours from 15 to 49 days of age had a 7% reduction in BWG and a 4% reduction in feed intake with no difference in FCR (Imik et al., 2012). Another group found a 25% reduction in BWG and a 21% reduction in feed intake with no difference in FCR when subjecting broilers to a cyclic HS for 10 hours per day then returning to TN temperature for the next 14 hours (Quinteiro-Filho et al., 2010). To better understand the variation in responses between cyclic and continuous HS, a meta-analysis was conducted on HS experiments from the literature comparing the difference in severity of effects on performance between cyclical and continuous HS. It was reported that continuous HS had a greater negative influence on feed intake (29% vs. 20%) and BWG (21% vs. 16%) (Schirmann et al., 2021).

Earlier HS studies investigated the effect of feed intake on performance of HS birds using a pair feeding strategy. This strategy was based on feeding a group of TN birds the same feed intake as a broiler under HS to determine what portion of the reduction in performance was associated with the decrease in feed intake. The results of this study indicated that about 63% of the growth depression in HS chickens can be attributed to the lower feed intake (Dale and Fuller, 1979). Another study demonstrated that continuous HS at 32°C resulted in a 16% reduction in BWG compared to the pair fed group consuming a similar amount of feed (De Souza et al., 2016). These studies would indicate that the loss of live performance is not only related to feed intake but rather there are several physiological factors including oxidative stress that are directly related to HS.

2.2.1.4 EFFECTS OF HEAT STRESS ON DIGESTIBILITY AND INTESTINAL HEALTH

Loss of performance could be associated to HS effects on digestibility and intestinal health. A previous study that compared the effects of a cyclic and continuous HS vs the control showed that continuous HS resulted in a reduction in dry matter, crude protein, and crude fat digestibility which was not associated with feed intake when comparing it to a pair fed group. However, broilers that were subjected to cyclic HS did not show a similar response (De Souza et al., 2016). An early study where broilers were subjected to a continuous HS at 32°C found an improvement in apparent metabolizable energy (AME) compared to the TN (Keshavarz and Fuller, 1980). Additionally, Rosa and coworkers (2007) found no difference in AME when subjecting broilers to a 32°C continuous HS at 31 days of age. These variations in responses could be due to the stage at which the birds were exposed to the stress as well as the duration of the stress which would have an impact on overall digestibility.

Reduced availability of oxygen and nutrients resulting from the diminished blood supply and reduced feed intake during HS can result in morphological changes and damage to the intestine caused by oxidative stress and inflammation. Quinteiro-Filho and coworkers (2012) demonstrated that cyclic HS for 10 hours per day at 31°C from 35 to 42 days increased intestinal permeability which resulted in the translocation of pathogens resulting in higher *Salmonella* counts in the liver and spleen. Other studies have reported a consistent reduction in villi to crypt ratio during HS which was associated with a reduced villi height and increased crypt depth which indicates less surface area for absorption of nutrients (Deng et al., 2012; Song et al., 2014; He et al., 2018). Heat stress can also cause major changes in the microflora of the gut such as a reduction in *Lactobacillus* and *Bifidobacterium* and reduction in coliforms and *Clostridium* (Song et al., 2014). These combined effects could impact the health of broiler chickens which would influence performance, digestibility, and livability.

2.2.1.5 EFFECT OF HEAT STRESS ON MEAT QUALITY AND FOOD SAFETY

Meat quality and food safety are two important factors in poultry production because they can directly affect consumer perception. In 1997, Mckee and Sams published a study where Nicholas tom turkeys were subjected to continuous HS at 32 °C at 17 weeks of age and processed at 21 weeks of age. The HS resulted in a faster decline in post-mortem pH which resulted in an increase of 0.4% drip and 5% cook loss compared to TN group. Another study conducted with Ross 308 broilers also reported a faster pH decline and less moisture when they were subjected to a continuous HS at 34 °C from 3 to 7 weeks of age compared to TN group (Aksit et al., 2006). Faster decline in pH is a result of the breakdown of glycogen to lactic acid during glycolysis post-mortem which results in the accumulation of lactic acid in muscle which has impact on water holding capacity and meat quality (Mckee and Sams, 1997). In another

experiment where Nicholas toms and hens were subjected to continuous HS at 30 °C from 16 to 20 weeks of age, there was no observed reduction in ultimate pH or cook loss (Owens et al., 2000). This indicates that the responses based on meat quality can be affected by elevated temperatures and could be associated with pH before processing.

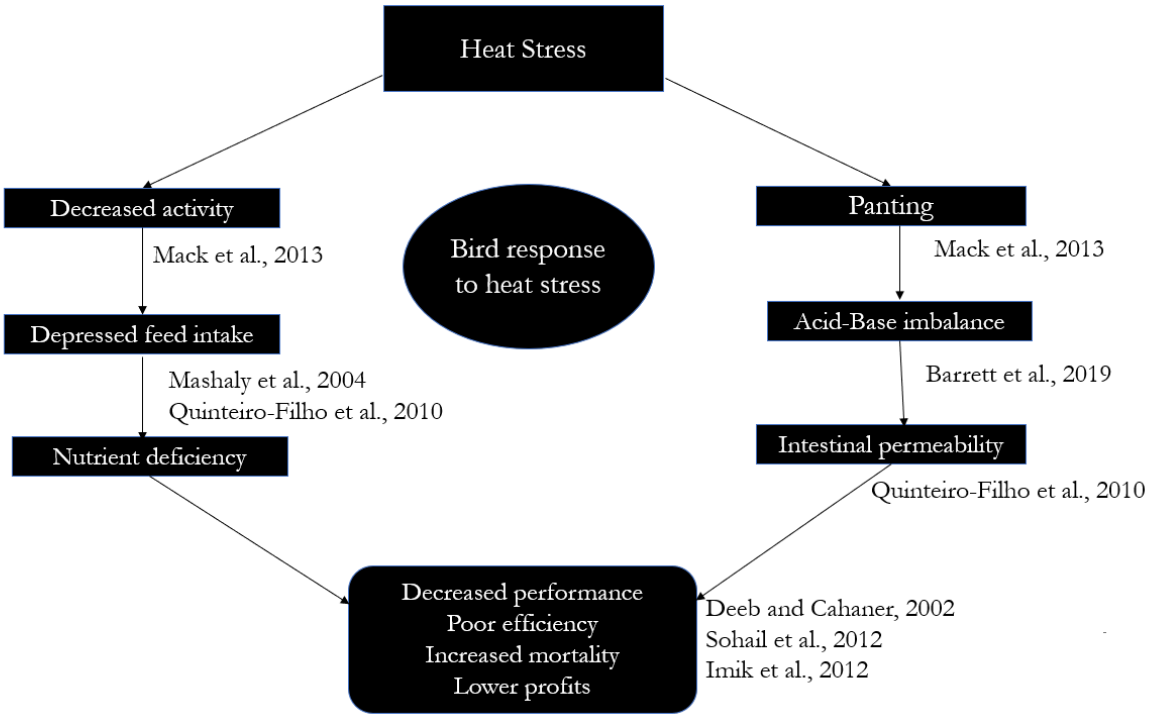

Figure 3. Summary of the impacts of heat stress on poultry production

2.3.3 STRATEGIES TO AMELIORATE THE EFFECT OF HEAT STRESS

2.3.3.1 NUTRITIONAL STRATEGIES

The growth rate and yield of commercial broilers can be suppressed by high dietary protein during HS (Cahaner et al., 1995). Although higher protein in poultry diets results in a higher heat increment that may be harmful during HS, feeding low protein diets causes them to consume more feed which results in increased heat production (Buyse et al., 1992). However, several experiments have eluded that there are beneficial effects of supplementing specific crystalline amino acids to diets during HS. Brake (1998) indicated that increasing the arginine:lysine ratio from 21 to 40 days improved FCR in broilers subjected to continuous HS at 31°C. In the same set of experiments, they reported that the in vitro uptake of arginine in the intestinal epithelium of broilers subjected to HS was decreased in the presence of lysine in the diet. This could be due to the role that arginine plays in vasodilation within the body. The uptake of crystalline amino acids was also reported to be affected by higher levels of sodium chloride and sodium bicarbonate (Balnave and Brake, 1999). An improvement in antioxidant function has been seen when supplementing sulfur amino acids to poultry diets (Willemson et al., 2011; Del Vesco et al., 2015). These responses are due to an increase in glutathione which is one of the most important antioxidants in the body counteracting effects of reactive species during HS.

2.3.3.2 FEED ADDITIVES

Vitamins and minerals have been studied extensively as a strategy to overcome HS. Vitamin C which functions as an antioxidant, showed beneficial effects on performance when broilers were subjected to two days of HS at 38°C (Pardue et al., 1985). The improved performance has been associated with a reduction in oxidative stress during HS. Sahin and coworkers (2001) reported that vitamin A, E, and zinc can ameliorate the negative effects of HS

in broilers which is due to a 1.2 to 1.5 times increase in antioxidants in the body. Phytochemicals such as cinnamon powder and curcumin have also been reported to have beneficial effects in broilers under HS conditions (Zhang et al., 2015; Sadeghi and Moghaddam, 2018). Both resulted in improved antioxidant status of broilers and better overall BW and FCR.

2.3.3.3 GENETICS

Genetic selection for increased growth rate and feed efficiency has improved the efficiency of the broiler industry to become the largest meat producer in the world. However, this selection has come with some unwanted consequences such as increased susceptibility to heat stress due to the higher growth rate (Deeb and Cahaner, 2001). Modern broilers have low heritability for heat tolerance and as a result, it was suggested that modern broilers should be selected under different environmental conditions (Mathur and Horst, 1994). Another strategy is the use of different major genes such as the naked neck gene or the frizzle gene. In the naked neck gene which causes a reduction in the feather mass (Merat, 1986). In the frizzle gene, the feathers tend to curl which reduces their size and they get less insulation (Yunis and Cahaner, 1999). These strategies have been used in tropical and subtropical countries for many years to reduce the impact of heat stress but not used in the other parts of the world due to reduced performance with these breeds.

2.3.3.4 HEAT CONDITIONING

One of the most promising strategies to create resistance to heat stress is early heat conditioning. Early heat conditioning is simply defined as exposing chickens to higher temperature early in life from 3 to 6 days for 24 h. Several studies have demonstrated that heat conditioning early will induce heat tolerance in broilers in market age (Arjona et al. 1988; Yahav

and Hurwitz, 1996). When exposed to higher temperatures at market age, the heat conditioned chickens demonstrate reduced body temperature, increased feed intake, and similar feed efficiency compared to control birds. The mechanism associated with this is the initiation of IGF-1 and proliferation, expression and activity of brush-border membrane enzymes which are crucial to the digestion of nutrients in the body (Uni et al, 2001). This suggests that heat conditioned chickens will be delayed in growth early in life but following that stress they can compensate the lag in growth overtime by consuming more feed.

2.3 DIRECT FED MICROBIALS

2.3.1 INTRODUCTION

Direct fed microbials are live microbial feed supplements that when administered in adequate amounts in the diet can improve microbial balance within the intestine (Fuller, 1989). In 2006, Europe banned the use of antibiotics in animal production due to increasing public concerns regarding bacterial resistance to antibiotics (Anadon, 2006). In the past several years, the poultry industry has slowly transitioned to antibiotic free production due to consumer demand. The result of this was to research products and strategies as alternatives to antibiotics. Previous studies have shown that DFMs can be utilized as alternatives to antibiotics (Applegate et al., 2010; Amerah et al., 2013). Direct fed microbials are categorized as *Bacillus*-based or lactic acid-producing bacteria. *Bacillus*-based DFMs are spore forming bacteria which means that they are thermostable and can survive at low pH. This is important when the high temperatures during feed processing and low pH during gastric digestion come to mind. Spores from *Bacillus*-based DFMs do not multiply in the intestine, thus these DFMs need to be fed continuously in the diet to maintain the microbial population. Lactic acid-producing bacteria on

the other hand are not spore forming which allows them to proliferate in the intestine and maintain the microbial population. However, these DFMs are not thermostable creating issues with survival during feed processing, lowering their efficacy.

2.3.2 EFFECT OF DIRECT FED MICROBIALS ON POULTRY PRODUCTION

2.3.2.4 MODIFICATION OF INTESTINAL MICROBIOTA

The diversity of intestinal microbiota plays an important role in metabolism, performance, digestibility, and health of broilers (Yadav and Jha, 2019). Infectious and non-infectious stressors can cause the microbiota to be altered which can result in increased inflammation and permeability in the intestine and reduction in digestibility resulting in performance losses (Wang et al., 2015). Supplementation of a *Bacillus*-based DFM to broiler and layer diets has resulted in positive effects on the microbiota by promoting beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* which are thought to be associated with improved performance (Knarreborg et al., 2008; Lei et al., 2014). *Bacillus* and *Lactobacillus*-based DFMs have the ability to reduce pathogens including *Clostridium perfringens* and avian pathogenic *Escherichia coli* (La Ragione et al., 2002; Knap et al., 2010). Froebel and coauthors (2020) found that administration of *Bacillus* cultures to Cobb broilers from 0 to 42 days of age resulted in a reduction in *Campylobacter* spp. in the cecum and an increase in *Lactobacillus*. Grimes and coworkers (2008) reported a one log reduction in *Salmonella* counts when supplementing a *Lactobacillus*-based DFM in diets to turkey poultry from 0 to 3 weeks of age. This indicates that DFMs not only support the intestinal balance for growth but can also improve microbial food safety since *Campylobacter* spp. and *Salmonella* are a major issue related to processed meats that go for human consumption.

2.3.2.1 NUTRIENT UTILIZATION

Bacillus based DFMs have been investigated previously as a principal source of microbial enzymes which aid in the digestion of nutrients within the body with many of them being effective in the production of amylases, proteases, lipases, and phytases (Lattorre et al., 2016). A previous study utilizing 2 doses of a *Bacillus amyloliquefaciens*-based DFM supplemented in the feed to Arbor Acre broilers from 0 to 42 days of age reported an improvement in dry matter, crude protein, and gross energy digestibility at 21 and 42 days of age (Lei et al., 2015). Another study utilizing Ross 708 broilers supplemented with a multi-strain *Bacillus* spp. DFM reported an improvement in apparent metabolizable energy when a reduction of 130 kcal/kg was applied to negative control diets (Nusairat and Wang, 2020).

The effects of the DFM on nutrient digestibility are more pronounced when there is a stressor within the environment. A previous study that challenged broiler chickens with *Escherichia coli* and supplemented with a *Bacillus subtilis* DFM reported a 3% improvement in crude protein digestibility (Manafi et al., 2016). This was correlated with an improvement in the villus height to crypt depth ratio as well as a reduction in coliforms, *Salmonella*, and *Escherichia coli* counts in the ceca. Jin and coworkers (2000) investigated the effect of *Lactobacillus* culture on enzyme activity in the small intestine of Arbor Acre broilers where they reported more amylase enzymes being secreted compared to the control but no increase in proteases or lipases. This indicates that different DFMs vary in the enzyme that they secrete.

2.3.2.2 GROWTH PERFORMANCE

The response to DFMs on growth performance varies depending on the level of challenge. Jin and coworkers (1998) fed Arbor Acre broilers a *Lactobacillus* culture at 3 different levels under tropical condition conditions and concluded that the two lower levels at

0.05 and 0.10% of the diet improved BWG by 100 g and reduced FCR by 12 points from 0 to 42 days of age. Furthermore, in 3 field trials that were conducted with Cobb broilers supplemented with *Lactobacillus* cultures in the water reported a 6-point improvement in FCR and 1.5% reduction in mortality (Timmerman et al., 2006) Another study that challenged broilers with necrotic enteritis observed an improvement in BWG and FCR when supplementing broilers with a *Bacillus*-based DFM (Hernandez-Patlan et al., 2019). These improvements were correlated with a reduction in IgA and intestinal permeability at 21 days of age. A previous experiment utilizing Cobb broilers challenged with live *Eimeria* and supplemented with a low, medium, and high level of *Bacillus*-based DFM resulted in a 2-point improvement in FCR but no difference in BWG (Johnson et al., 2020). The DFM also resulted in improved livability from 0 to 42 days of age. In a previous study where Ross 308 broilers were fed a *Lactobacillus*-based DFM without a challenge, there was no observed improvement on any performance parameters compared to the control. However, the DFM was still able to improve the villus height to crypt depth ratio in the ileum (Awad et al., 2009). This indicates that the level of challenge would dictate the response of broilers to the DFM.

2.3.2.3 INTESTINAL INTEGRITY

Improvements in intestinal morphological measurements can indicate better nutrient digestibility due to the increase in surface area for nutrient absorption. DFM inclusion in the feed has been shown to improve intestinal histomorphology. Jin and coworkers (2000) reported an increase in villus height and villus height to crypt depth ratio when supplementing a *Lactobacillus* DFM to Arbor Acre broilers. In another experiment where broilers were subjected to HS, Song and coworkers (2014) reported that a *Bacillus*-based DFM improved villus height, crypt depth, and villus height to crypt depth ratio. This was correlated with a higher expression

of tight junction protein which protects the intestinal barrier contents from leaking into circulation and causing disease. Furthermore, a study done investigating the effect of a *Lactobacillus*-based DFM on male broilers from 0 to 42 days of age reported an improvement in villus length and increase in goblet cell density with the supplementation of the DFM compared to the control (Aliakbarpour et al., 2012). Goblet cells are important because they secrete mucin which can further exclude pathogens from adhesion to the intestinal epithelium (Shroyer et al., 2011). Furthermore, DFMs can also maintain intestinal integrity through reduction of intestinal permeability. Adhikari and coworkers (2019) reported that a *Bacillus*-based probiotic was able to reduce the intestinal permeability in broiler chickens 10 days post-challenge with *Salmonella enteriditis.* A recent study also reported a reduction in IgA and intestinal permeability at 21 days of age with the supplementation of a *Bacillus*-based DFM to broiler chicks from 0 to 21 days of age. These findings indicate that DFMs improve intestinal integrity through several mechanisms that result in improved performance and overall health.

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Scientific section: Metabolism and Nutrition

CHAPTER 3: TRYPTOPHAN REQUIREMENT OF FIRST-CYCLE COMMERCIAL LAYING HENS IN PEAK EGG PRODUCTION

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ABSTRACT

An experiment was conducted to evaluate the digestible tryptophan (Trp) requirement of first cycle laying hens from 22 to 34 weeks of age. A total of 252 Hy-line W-36 laying hens were selected at 16 weeks of age and allocated by weight (P = 0.90) to seven dietary treatments resulting in 12 replicate cages of three birds for each treatment. A Trp deficient basal diet was formulated using corn, corn gluten meal, and soybean meal for each of the 3 dietary phases and supplemented with synthetic L-Trp to generate diets that provided 105, 119, 133, 147, 162, 176, and 190 mg digestible Trp on a daily basis over the experimental period based on feed intake. To adapt the hens to experimental diets, pullets were fed complete diets that contained increasing amounts of corn gluten meal. Hens were limit-fed a similar amount of experimental diets based on feed intake expected under commercial conditions. Linear and quadratic broken-line, and quadratic polynomial models were used to estimate digestible Trp requirements based on hen-housed egg production (HHEP), egg mass (EM) and feed efficiency (FE). FE was calculated using EM and feed intake. Digestible Trp requirements were estimated to be 137, 183 and 192 mg/d for HHEP; 133, 180 and 183 for EM and 133, 177 and 173 for FE using linear broken-line, quadratic broken-line and quadratic polynomial analysis, respectively. The quadratic broken line model in this experiment resulted in the best fit (R^2) for all parameters measured. Linear broken line estimates resulted in lower estimates that the other models, and HHEP resulted in higher estimated digestible Trp requirement than EM and FE.

Key words: digestible tryptophan, egg production, laying hen, requirement, ratio

INTRODUCTION

Tryptophan is an essential amino acid and third limiting in typical commercial corn-soybean diets for white egg laying hens (Russell and Harms, 1999). Tryptophan is primarily used for protein synthesis to maintain egg production, but also plays a role as a precursor of serotonin which is synthesized to melatonin to regulate sleep, appetite, and stress responses. Additionally, Trp can be converted to the B-vitamin niacin through the kynurenine pathway (Rogers and Pesti, 1990). Genetic selection has resulted in an annual improvement in egg production of 2 to 3 eggs and 1-to-3-point reduction in feed efficiency in laying hens from 2010 to 2015 (Preisinger, 2018). This yearly improvement in productivity requires the reevaluation of amino acids requirements on an ongoing basis in order to support the improved performance. Russell and Harms (1999) used 53 to 59 wk old Hy-Line W-36 laying hens to estimate a 136.5 mg/d Trp requirement when hens consumed 97 g/d of feed. Using younger 28 to 36 wk old Hy-Line W-36 laying hens, the same group estimated a 149 mg/d Trp requirement with 95 g of daily feed intake. More recently, 41 to 60 wk old Hy-Line W-36 laying hens were estimated to have a 153 mg/d Trp requirement when the hens consumed 97 g/d of feed (Wen et al. 2019). Several other authors have estimated requirements of laying hens using the Trp:Lysine ratio applying the ideal protein concept. Bregendahl et al. (2008) reported a Trp requirement of 120 mg/d of Trp relative to 482 mg/d of Lys based on broken line estimates using Hy-Line W-36 hens from 28 to 34 weeks of age. A more recent publication estimated a higher requirement of 151 mg Trp/d relative to 675 mg/d of Lys based on 95 g/d intake of Hy-Line W-36 hens from 30 to 36 weeks of age (Mousavi et al., 2017). However, none of the previous experiments have investigated the requirement of Trp as the pullets are transitioning into egg production. Therefore, the objective of the current experiment was to determine the Trp requirement of first cycle laying hens from

22 to 34 weeks of age based on hen-housed egg production (HHEP), egg mass, and feed efficiency using linear broken line, quadratic broken line, and quadratic polynomial models.

MATERIALS AND METHODS

DIET FORMULATION

The Trp-containing ingredients (i.e., corn, soybean meal, and corn gluten meal) were analyzed for amino acid concentrations by wet chemistry (AOAC method 982.03, Eurofins, Des Moines, IA, 50321) prior to dietary formulation. Up to 7% corn gluten meal was used to replace soybean meal in the diet to generate the Trp deficient diets. Low concentrations of 2 to 4% corn gluten meal were fed gradually starting in the pullet phase to slowly acclimate the pullets/hens and reduce any potential reductions in feed intake due to transitioning to experimental diets. All experimental diets were mixed using a basal diet that was analyzed for Trp (AOAC method #988.15, Eurofins, Des Moines, IA, 50321) before final experimental diet generation. The basal diets were formulated to be adequate in all essential nutrients with the exception of Trp (Tables 1 and 2; Hy-Line International, 2018). The basal diet was then split into equal aliquots to generate the 105, 119, 133, 147, 161, 175, and 190 mg per day of digestible Trp diets via supplementation with synthetic L-Trp (98% feed grade, CJ Bio, PT. Cheil Jedang, Indonesia). (Table 3). Since the feed intake increases weekly as the hens are coming into production, diets were formulated and mixed every 2 weeks to meet the nutrient demands of the hens based on their feed consumption at each time point which increased up to 95 g/d intake. Diets were formulated and adjusted to maintain the same milligrams per day of Trp intake regardless of feed intake due to changes in the requirement in percentage as the feed intake increases. At the

conclusion of the experiment, pooled samples of experimental diets were sent to Eurofins (Des Moines, IA, 50321) for amino acid analysis.

ANIMALS AND HOUSING

All animal work was approved by the Institutional Animal Care and Use Committee. In total, 252 Hy-Line W-36 laying hen pullets were selected at 16 wk of age from a pullet flock of 500 hens and allocated to cages to have similar cage body weight (BW 1,532 g; SE = 10; P = 0.90). The 7 dietary treatments were randomly assigned to cages resulting in 12 replicate cages of 3 hens/cage (464.5 cm^2/cage) utilizing two A-frame battery cage units. An environmentally controlled dark-out room was used to house birds. The temperature was set to be maintained between 68-75°F throughout the duration of the experiment although due to summer heat conditions, temperatures reached a maximum 80°F for short time periods (4 afternoon time periods). Hens were limit-fed a schedule of feed of 85, 90, 95 g/hen/d from 22 to 24, 24 to 26, 26 to 34 weeks of age. Hens were provided ad libitum access to water throughout the experiment. Experimental diets were provided starting at 16 wk of age to pre-test hens before data analysis began at 22 wk of age when consistent egg production was initiated.

Laying hens were monitored twice daily throughout the duration of the experiment and mortality were noted, weighed, euthanized, and removed as they occurred. Feed allocation for the two-wk period was weighed into individual labeled feed buckets for each replicate cage and daily allocation was provided at approximately 9 am from each individual feeding bucket. At the end of the two-wk period any remaining feed in the feed tray was returned to the corresponding feed bucket and weighed to determine feed intake. Eggs were counted, weighed, and collected by cage between 09:30 and 10:30 daily. Egg weights and numbers were used to calculate HHEP

and egg weight. The HHEP was calculated using the number of eggs per 2 week period divided by the number of hen days during the week. Egg mass was calculated from egg weight and HHEP. FE was calculated using EM and feed intake. Hens were weighed by cage every 4 weeks.

STATISTICAL ANALYSIS

Performance data including HHEP, feed intake, egg weight, egg mass, body weight, and FE were analyzed using a one-way ANOVA with means separated by repeated measures using a Tukey's adjustment in JMP 14 (SAS Institute Inc., Cary, NC). Significance was accepted at $P \leq 0.05$. Digestible Trp intake per hen was calculated from the feed intake and analyzed Trp values by multiplying the feed intake by the percent. Analyzed total Trp values were converted to digestible Trp values using AminoDat Software (Version 5, 2016) by multiplying the digestibility coefficient of each ingredient by the amount of Trp provided by that ingredient in the diet. These values were then used to determine the digestible Trp requirement for laying hens using linear broken line, quadratic polynomial, and quadratic broken line analyses for each of HHEP, EM, and FE. Regression analysis was performed using the non-linear model analysis in JMP 14 (SAS Institute Inc, Cary, NC).

RESULTS AND DISCUSSION

LAYING HEN PERFORMANCE

Egg production ranged from 86.2% to 93.9% and generally increased until 147.5 mg digestible Trp after which the data appeared to plateau (Table 3). Although there were no statistical differences among diets of 148 mg/d and above, there was a 1.7 percentage point increase in egg production compared to deficient Trp diet. These results are consistent with previous research (Harms and Russell, 2000; Wen et al., 2019), while others found that egg

production followed a quadratic pattern where egg production began to decrease at 0.19% and 0.20% or 210 mg/d and 181 mg/d, respectively (Jensen et al., 1990; Cardoso et al., 2014). As expected, low digestible Trp diets had a 7.7 percentage point reduction in egg production compared to the diets with the highest dietary digestible Trp concentration. Average BW ranged from 1,481 to 1,536 g which is consistent with the Hy-Line W-36 performance objectives at 34 weeks of age. There was a tendency ($P = 0.06$) for a dose response of Trp concentration on BW as the hens fed the lowest concentrations of Trp had the lowest BW. In a previous report, Wen et al. (2019) reported a quadratic response to BW loss where the BW loss was lowest with the 169 mg/d inclusion of Trp, then increased with additional supplementation. However, when laying hens were fed diets containing 145 mg/d Trp or above (Trp:Lys ratio of 17) there was no observed BW loss (Bregendahl et al., 2008). These differences might be due to differences in time of experiments as the longer experiments would have more time for differences in BW to manifest. Feed intake over the 22- to 34-wk period ranged from 91.7 to 93.1 g/d. Overall there were few differences across the range of digestible Trp diets (feed intake across the 7 treatment diets were generally within 1 g). Differences in feed intake among treatments were only observed between the lowest two concentrations of Trp where hens fed 119 mg/d had higher feed intake than those fed 105 mg/d ($P \leq 0.05$). These data are inconsistent with previous reports that found feed intake was reduced with deficient concentrations of Trp (Russell and Harms, 1999; Wen et al., 2019). This could be due to the transition period used in this experiment to reduce effects on feed intake.

Although Trp in the diet had a significant effect on the egg weight ($P \leq 0.01$), no clear pattern was observed, and the biological relevance of this response is questionable. The literature is also inconsistent regarding the effect of Trp on egg weights with reports of a dose

response effect on egg weights (Russell and Harms, 1999; Wen et al., 2019) and other reports (Jensen et al., 1990; Cardoso et al., 2014) without differences in egg weights with various dietary Trp concentrations.

Egg mass values ranged from 46.7 to 50.2 g, which is consistent with Hy-Line W-36 breed objectives at this age. Egg mass and feed efficiency responded similarly as both increased until approximately the 162 mg/d Trp treatments with no additional improvements above that treatment ($P \leq 0.05$). These results are generally consistent with egg mass and FE results from previous reports (Bregendahl et al., 2008; Cardoso et al., 2014; Wen et al., 2019).

TRYPTOPHAN REQUIREMENT

The linear broken line model resulted in estimates of 137, 133, and 133 mg/d of digestible Trp for HHEP, egg mass, and feed efficiency, respectively (Table 5; Figure 1). The linear broken line analysis is the classic method of modeling requirements of amino acids defined by Morris and Jennings (1973). The model is based on an increasing or decreasing slope portion where the response is increasing with every increasing supplementation of a nutrient because the animal is below requirement for that specific nutrient, however, after the animal reaches the requirement, the result is a straight-line plateau without a slope. The point at which that plateau starts, or the line breaks is the maximum response and requirement estimate. In this experiment, the linear broken line method estimated the lowest Trp requirements compared to the other models which is consistent with previous estimations of amino acid requirements in the literature (Morris and Jennings, 1973; Wen et al., 2019). Russell & Harms (1999) found the requirement for Trp to be 136 mg/d of total Trp or 107 mg/d of digestible Trp for Hy-line W-36 laying hens from 53 to 59 wks of age. In another study, Harms & Russell (2000) found the requirement for HHEP to be 140 mg/d of total Trp which would be estimated to 110 mg/d of

Digestible Trp for Hy-line 36 laying hens from 28 to 36 wks of age. Lastly, the most recent experiment investigating Trp requirement in Hy-line W-36 hens from 41 to 60 wks of age determined a requirement of 153, 156, and 140 mg/d of total Trp for HHEP, egg mass, and FE (Wen et al., 2019). These requirements can be converted to 120, 122, and 110 mg/d of digestible Trp. Contrasting historical results with current findings, the Trp requirement for laying hens coming into production might be higher to support the peak production of laying hens when egg production is at the highest output and efficiency compared to post-peak. Additionally, Trp is needed along with other amino acids to support the growth and development of hens to sustain and support egg production for longer periods of time.

Although the linear broken-line model is the classical requirement method, others have however contended that the idea is not to formulate to a requirement for maximum performance but instead to provide the nutrients for a maximum profit (Pesti et al., 2009). Therefore, a variety of models can be used to estimate further responses that the linear broken line does not incorporate. Quadratic polynomial analysis has also been widely used to estimate requirements based on the concept of "diminishing marginal productivity" where the effect of every addition of a nutrient is diminished until you reach the requirement (Pesti et al., 2009). However, the issue that is faced with quadratic polynomial is that it lacks a clear plateau for determining requirements and after the maximum response is achieved, the response falls rapidly which is not consistent with biological data (Pesti et al., 2009). Furthermore, there are differences in where the requirement should be estimated as some reports have used 95% of the maximum response (Wen et al., 2019) while others (Pesti et al., 2009; Cardoso et al., 2014) have used the maximum response. The quadratic polynomial resulted in estimates of 192, 183, and 173 mg/d of digestible Trp at 95% of the maximum response for HHEP, egg mass, and feed efficiency, respectively

(Figure 2). In Hy-line W-36 laying hens from 41 to 60 wks, the quadratic polynomial estimates were 180, 187, and 182 mg/d of total Trp or 142, 147, and 143 mg/d of digestible Trp for HHEP, egg mass, and FE, respectively (Wen et al. 2019). Other authors have explored modeling Trp requirements based on the ratio to lysine using the linear broken line (Bregendahl et al., 2008) or quadratic polynomial model (Lima et al., 2012; Cardoso et al., 2014). The digestible Lys intake of the laying hens in this experiment was 800 mg/d, consistent with previous Lys requirement estimates for laying hen egg production and egg mass, 727 mg/d to 846 mg/d (Pastore et al., 2018; Spangler et al., 2019). The estimates for Trp:Lys ratio in this experiment using the linear broken line were 17.1, 16.6, and 16.6 for egg production, egg mass, and feed efficiency, respectively. This estimate is lower than the previous report of 24.6, 24.5, and 22.3 Trp:Lys ratios found in Hy-Line W-36 laying hens from 28-34 weeks of age (Bregendahl et al., 2008). Estimates from the quadratic polynomial were 24.0, 22.9, and 21.6 Trp:Lys for egg production, egg mass and feed efficiency, respectively. These results are in agreement with previous reports of estimated requirements of 23.9 to 25.4 Trp:Lys for egg production, egg mass and feed efficiency (Lima et al., 2012; Cardoso et al., 2014).

The quadratic broken line includes the advantages of a plateau region of the linear broken line and the diminishing marginal productively of the quadratic polynomial. The requirement can be estimated at the point where the line breaks in the same manner as the linear broken line analysis (Pesti et al. 2009). The estimates based on the quadratic broken line were 183, 180, and 177 mg/d of digestible Trp for HHEP, egg mass, and feed efficiency, respectively (Figure 3). These estimates were higher than the estimates for the linear broken line, but they were slightly lower or similar to quadratic polynomial estimates. In comparison, Wen et al. (2019) found the requirement of total Trp to be 182, 200, and 164 mg/d or 143, 157, and 129 mg/d of digestible

Trp for HHEP, egg mass, and FE. Overall, results from all models indicate that laying hens coming into production have a higher requirement for Trp to satisfy the requirements of egg production while maintaining and possibly increasing their body weight. Furthermore, there seems to have been an increase in Trp requirements due to genetic selection that can be observed when comparing these results with the findings of Russell and Harms (1999).

In previous Trp requirement studies where gelatin or corn gluten meal were used to generate Trp deficient diets, there was an associated drop in feed intake after the transition to experimental diets that were formulated to be sufficient in dietary Trp (Table 6). The introduction of gelatin to experimental diets caused feed intake to drop to 82 to 86 g compared to an expected intake of 97 g/d (Russell and Harms, 1999). Corn gluten meal in experimental diets without an adaptation period also resulted in a drop of 11-17 g/d drop in feed intake in the first 6 weeks (Wen et al., 2019). However, the current experiment resulted in no reduced feed intake in Trp sufficient diets after the transition to experimental diets. This was largely due to the adaptation of pullets to experimental diets by adding corn gluten meal in stages during the pullet phase of production.

The current experiment indicated a higher Trp requirement than previously seen in the literature as the hens are transitioning into egg production to support the increased egg production up to peak. The quadratic broken line model in this experiment resulted in the best fit (R^2) for all parameters measured compared to the other two models. The adaptation period prior to the start of this experiment allowed for a more accurate estimation of requirements since no negative effect on feed intake were observed in the transition to experimental diets. For maximizing egg production, the optimum intake of digestible Trp was 137, 183, and 192 based on the linear broken line, quadratic broken line, and quadratic polynomial, respectively.

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Table 1. Formulation of Trp-deficient basal diets fed to 22 to 34-week-old Hy-Line W-36 laying hens.

Ingredient	Peak 22-24 wks of age	Peak 24-26 wks of age	Peak 26-34 wks of age
	(%)		
Corn	68.19	68.29	70.92
Corn gluten meal	7.00	7.00	7.00
Soybean meal	11.00	11.00	8.83
Soy oil	0.70	0.70	0.32
Salt	0.20	0.20	0.20
Sodium bicarbonate	0.13	0.13	0.13
L-Methionine	0.33	0.30	0.30
L-Lysine•HCl	0.57	0.50	0.51
L-Threonine	0.21	0.21	0.20
L-Valine	0.26	0.26	0.22
L-Isoleucine	0.23	0.23	0.23
L-Tryptophan	0.00	0.00	0.00
L-Arginine	0.32	0.32	0.27
Oyster shell (large particle)	4.69	4.69	4.69
Limestone (small particle)	4.69	4.69	4.69
Dicalcium phosphate	0.87	0.87	0.88
Phytase[1]	0.01	0.01	0.01
Choline chloride (60% Choline)	0.10	0.10	0.10
Vitamin premix[2]	0.50	0.50	0.50
Total	100.00	100.00	100.00

[1] Quantum Blue (300 FTU/kg) was formulated to provide 0.12% of calcium and nonphytate phosphorus

[2] Provided per kilogram of diet: vitamin A, 6595 IU; vitamin D_3, 2209 ICU; vitamin E, 1.65 IU; vitamin B_{12}, 6.60 µg; menadione, 1.15 mg; riboflavin, 4.12 mg; D-pantotheic acid, 6.07 mg; niacin, 19.79 mg; choline, 381.68 mg; Co, 0.25 mg; Cu, 4.04 mg; I, 1.00 mg; Fe, 50.65 mg; Mn, 64.26 mg; Zn, 48.69 mg.

Table 2. Nutrient profile of Trp-deficient basal diets fed to 22 to 34-week-old Hy-Line W-36 laying hens. [1, 2]

Nutrient	Peak (22-24 weeks of age)		Peak (24-26 weeks of age)		Peak (26-34 weeks of age)	
	Calculated	Analyzed	Calculated	Analyzed	Calculated	Analyzed
	(%)					
Crude protein	16.19	16.18	16.20	16.05	15.30	15.45
ME, kcal/kg	2,954	---	2,954	---	2,951	---
Calcium	3.90	---	3.90	---	3.90	4.03
Nonphytate P	0.30	---	0.30	---	0.30	0.33
Fat	3.70	---	3.70	---	3.42	3.93
Met	0.61 (0.59)	0.56	0.58 (0.56)	0.60	0.58 (0.55)	0.54
Cys	0.26 (0.22)	0.24	0.26 (0.22)	0.25	0.26 (0.21)	0.23
Met+Cys	0.88 (0.82)	0.80	0.85 (0.79)	0.85	0.85 (0.77)	0.77
Lys	1.03 (0.96)	1.04	0.98 (0.91)	1.04	0.98 (0.86)	0.97
His	0.39 (0.29)	0.38	0.39 (0.29)	0.41	0.39 (0.28)	0.37
Trp	0.15 (0.12)	0.16	0.15 (0.12)	0.16	0.15 (0.11)	0.14
Thr	0.78 (0.69)	0.74	0.78 (0.69)	0.78	0.78 (0.66)	0.71
Arg	1.08 (1.01)	0.98	1.08 (1.01)	1.05	1.08 (0.90)	0.87
Ile	0.81 (0.76)	0.77	0.80 (0.76)	0.85	0.81 (0.73)	0.75
Leu	1.79 (1.65)	1.75	1.79 (1.65)	1.87	1.79 (1.60)	1.70
Val	0.94 (0.87)	0.91	0.94 (0.87)	0.99	0.90 (0.80)	0.86
Gly	0.55 (0.50)	0.55	0.55 (0.50)	0.59	0.55 (0.47)	0.52
Ser	0.77 (0.54)	0.77	0.77 (0.54)	0.81	0.77 (0.54)	0.68

[1] Diets were formulated to the same nutrient intake on a daily basis and differences in formulation were to account for feed intake. Basal and experimental diets were mixed every two weeks and analysis of basal diets was on a pooled basis if diets were fed for more than two weeks.

[2] Calculated amino acid values are reported as total dietary amino acid (digestible amino acid) and analyzed amino acid values are reported as total dietary amino acid.

Table 3. Formulated and analyzed digestible Trp content of experimental diets fed to 22 to 34 wk old laying hens.[1]

Dietary digestible Trp	Peak (22-24 weeks of age)		Peak (24-26 weeks of age)		Peak (26-34 weeks of age)	
	Calculated	Analyzed	Calculated	Analyzed	Calculated	Analyzed
Mg/d	(%)					
105	0.148 (0.123)	0.16 (0.13)	0.149 (0.116)	0.15 (0.12)	0.136 (0.110)	0.14 (0.12)
119	0.165 (0.140)	---	0.157 (0.132)	0.16 (0.13)	0.155 (0.125)	0.16 (0.13)
133	0.181 (0.156)	0.17 (0.14)	0.173 (0.148)	---	0.165 (0.140)	0.16 (0.13)
147	0.198 (0.173)	---	0.189 (0.164)	0.18 (0.15)	0.185 (0.155)	0.19 (0.16)
162	0.215 (0.190)	0.20 (0.17)	0.204 (0.179)	---	0.198 (0.170)	0.20 (0.17)
176	0.232 (0.207)	---	0.220 (0.195)	0.21 (0.17)	0.209 (0.185)	0.20 (0.17)
190	0.249 (0.224)	0.22 (0.18)	0.236 (0.211)	---	0.226 (0.200)	0.22 (0.18)

[1] Supplemental L-Trp was added to the basal diet to generate final experimental diets that contained 104.50, 118.75, 133.00, 147.25, 161.50, 175.75, and 190.00 mg per day of digestible Trp based on projected feed intake.

[2] Calculated amino acid values are reported as total dietary amino acid (digestible amino acid) and analyzed amino acid values are reported as total dietary amino acid.

Table 4. Body weight (BW), hen-housed egg production (HHEP), feed intake (FI), egg weight (EW), egg mass (EM) and feed efficiency (FE) of 22-34 wk old laying hens fed 104.50, 118.75, 133.00, 147.25, 161.50, 175.75, and 190.00 mg digestible Trp per day.[1]

Dietary Trp	BW	HHEP[2]	FI	EW	EM	FE
(mg/d)	(g)	(%)	(g/d)	(g)	(g)	(g:kg)
105	1,481	86.3^{c}	91.7^{b}	54.3^{a}	46.7^{b}	510^{c}
119	1,489	90.8^{ab}	93.2^{a}	53.4^{b}	48.4^{ab}	519^{bc}
133	1,495	89.1^{bc}	92.7^{ab}	54.3^{a}	49.8^{a}	531^{ab}
147	1,515	92.3^{ab}	92.7^{ab}	53.4^{b}	49.2^{a}	538^{a}
162	1,519	92.6^{a}	92.9^{ab}	54.0^{ab}	50.2^{a}	539^{a}
176	1,536	93.7^{a}	92.5^{ab}	54.6^{a}	49.9^{a}	540^{a}
190	1,517	94.0^{a}	92.6^{ab}	53.2^{b}	50.0^{a}	540^{a}
Pooled SEM	43	0.79	0.28	0.32	0.53	4.5
ANOVA	P value					
Treatment	0.06	**≤ 0.01**	**0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**
Week	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**
Trt x Week	0.67	0.99	0.98	0.99	0.99	0.99

[1] Initial BW = 1,532 g ($P = 0.65$)

[2] Overall mortality was negligible, with one mortality from hens fed 133.00 mg D Trp/d.

[a-c] Means within a column that do not share a common superscript differ ($P \leq 0.05$).

Table 5. Trp requirement (mg/d) of 22 to 34-wk-old Hy-Line laying hens estimated using linear broken-line, quadratic broken-line, and quadratic polynomial models based on hen-housed egg production (HHEP), egg mass (EM) and feed efficiency (FE).

	Model	Trp Requirement	Ordinate value	Regression equation	R^2
HHEP (%)	Linear broken line	137	93.3	$y = 93.3 - 0.1 (x - 136.6)$	0.83
	Quadratic broken line	183	93.5	$y = 93.5 - 0.0009 (x – 183.3)^2$	0.96
	Quadratic polynomial	192	93.4	$y = 61.7 + 0.3x - 0.0009x^2$	0.78
EM (g/d)	Linear broken line	133	50.1	$Y = 50.2 - 0.1 (x - 133.0)$	0.77
	Quadratic broken line	180	51.2	$y = 51.2 - 0.0004 (x – 180.0)^2$	0.96
	Quadratic polynomial	183	51.2	$y = 32.3 + 0.2x - 0.0006x^2$	0.84
FE (g/kg)	Linear broken line	133	540.0	$y = 540.0 - 0.8 (x - 132.5)$	0.81
	Quadratic broken line	177	543.8	$y = 543.8 - 0.006 (x – 177.4)^2$	0.96
	Quadratic polynomial	173	541.6	$y = 386.4 + 1.7x- 0.005x^2$	0.82

Table 6. Effects of laying hen feed intake on Trp requirement when gelatin or corn gluten meal are introduced into Trp sufficient experimental diets with or without an adaptation period.

Reference	**Adapted**	**Time**	**Feed intake**	**Expected feed intake**
		(Weeks)	(g)	
Russell and Harms (1999)	No	53 to 59	82-86	97
Russell and Harms (2000)	No	28 to 36	90	95
Current experiment	Yes[1]	22 to 28	87	88

[1]Adapted starting in the pullet phase for 8 weeks with graded levels of corn gluten meal increased on a 2-week basis

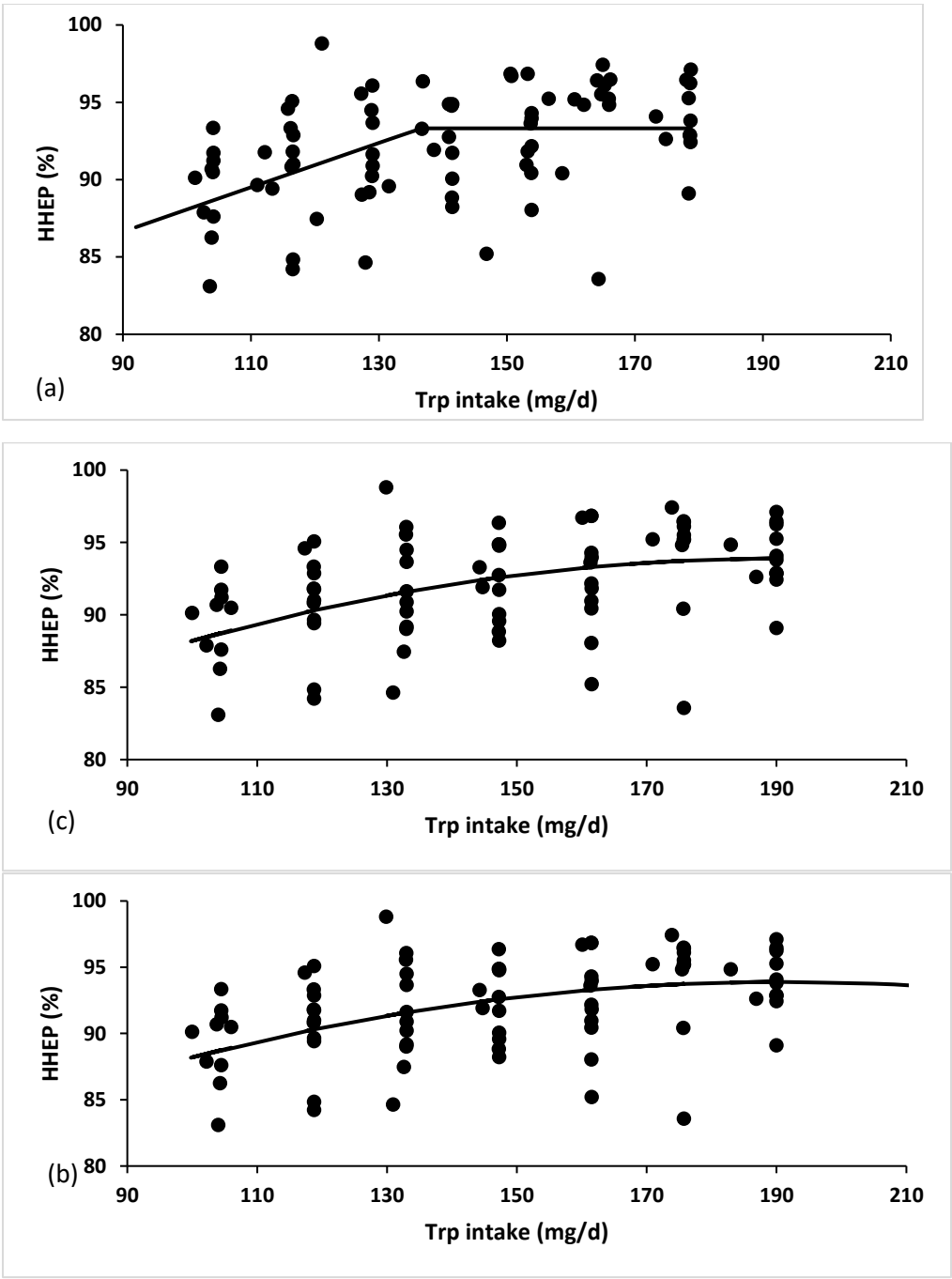

Figure 1. Digestible Trp requirement of 22 to 34-week-old Hy-Line W-36 laying hens estimated using (a) linear broken line regression; $y = 93.3 - 0.1 (x - 136.6)$; $R^2 = 0.83$, (b) quadratic polynomial regression; $y = 61.7 + 0.3x - 0.0009x^2$; $R^2 = 0.78$, and (c) quadratic broken line regression; $y = 93.5 - 0.0009 (x – 183.3)^2$; $R^2 = 0.96$ for hen-housed egg production (HHEP; %)

CHAPTER 4: LYSINE AND SULFUR AMINO ACID REQUIREMENTS OF BROILER CHICKS OVER SHORT TIME PERIODS WITHIN THE STARTER PHASE.

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SUMMARY

Six experiments were conducted to validate the hypothesis that Lys and SAA requirements decrease within the starter phase using 3-day periods from 2 to 11 d of age. In the first 3 experiments, 7 diets were generated by adding L-Lys to a lysine deficient basal diet in 0.10% increments, ranging from 0.85 to 1.45% Digestible Lys. In Experiments 4 to 6, 7 diets were generated by adding in increments of 0.07% DL-methionine to a SAA deficient diet to produce diets ranging from 0.63 to 1.04% SAA. The linear broken line estimate for digestible Lys was 1.22, 1.17 and 1.16% for BWG and 1.31, 1.21, and 1.14% for FCR in experiments 1, 2, and 3, respectively. The linear broken line estimate for SAA was 0.82, 0.81 and 0.94% for BWG and 0.82, 0.80, and 0.90 for FCR in experiments 4, 5, and 6, respectively. These results indicated that Lys requirements decreased linearly as hypothesized, however, the SAA requirements did not follow same pattern.

DESCRIPTION OF THE PROBLEM

The starter phase of production is a crucial stage of production requiring access to feed with high concentrations of dietary amino acids due to low feed intake. This increase in amino acids is important to support the growth and development of the systems and organs of the chicks needed to allow for the rapid muscle growth later in life (Schmidt et al., 2009). Dietary amino acid concentrations decrease as birds are transitioned to grower and finisher phases due to increased feed intake. Traditionally experiments have focused on requirement estimations that span an entire phase (Kalinowski et al., 2003; Garcia et al., 2005; Dozier et al., 2010; Cemin et al., 2017). However, Emmert and Baker (1997) introduced and validated the concept of using regression equations to predict short term requirements within each phase to smooth out the large drops in requirements that would be seen during the transition from starter to grower and finisher

diets which would ultimately result in diet savings. In addition, feed disturbances could be minimized by avoiding large changes in diets needed to reduce cost from one phase to the next (Saleh et al., 1997). This was accomplished by changing diets every 1 to 2 days to follow the requirements of the birds more closely according to their age. However, the logistics of formulating and producing feed as well as the transportation of feed every 2 to 3 days would not be ideal or economically viable. To solve this issue, authors suggested producing a high and low nutrient dense diet and placing them in two different bins on the farm then mixing them in different proportions to meet the requirements of broilers based on the regression equations (Pope et al., 2002). However, this concept was put forth with the assumption that amino acid requirements will decrease within each phase without direct validation (Pope et al., 2002). Therefore, the objective of these experiments was to validate the hypothesis that SAA and Lys requirements decrease with age and to determine the Lys and SAA requirements within the starter phase (2 to 11 d of age) over 3-day periods.

MATERIALS AND METHODS

DIET FORMULATION

Two starter diets were formulated to be deficient in either Lys or SAA and used as a basal diet to create experimental diets. The Lys deficient diet was formulated to contain 0.85% digestible Lys using corn, corn gluten meal, DDGS, and soybean meal as the major amino acid containing ingredients (Tables 1 and 2). The SAA deficient diet was formulated to 0.63% digestible SAA via corn, soybean meal, and poultry byproduct meal (Table 3 and 4). The basal diets were formulated to be adequate in all essential nutrients according to Ross 708 broiler nutritional recommendations with the exception of Lys or SAA in experiments 1 to 3 and 4 to 6,

respectively (Aviagen, 2018). The basal diets were then split into equal aliquots. Lysine-HCL was added at 0.10% increments to generate experimental diets that contained 0.85%, 0.95%, 1.05%, 1.15%, 1.25%, 1.35%, and 1.45% Lys and DL-Met was added to the basal diet at 0.07% increments to generate experimental diets that contained 0.63%, 0.70%, 0.77%, 0.83%, 0.90%, 0.97%, and 1.04% SAA. Basal and experimental diets were sampled when diets were removed from the mixer and a composite feed sample was used for amino acid composition analysis (University of Missouri AESCL, Columbia, MO 65211). Additional starter diet was formulated and manufactured according to Ross 708 broiler nutritional recommendations (Aviagen, 2018) and used to feed all chicks before they were transitioned to experimental diets at 2, 5, and 11 days of age. Diets in all experiments were manufactured in a mash form.

BROILER MANAGEMENT

All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech (Blacksburg, VA). Six independent experiments were conducted for estimating the Lys and SAA requirements from 2 to 5, 5 to 8, and 8 to 11 days of age. In experiment 1 to 3 and 4 to 6, a total of 1250 male Hubbard x Ross 708 chicks were obtained from a commercial hatchery at day of hatch and placed in battery. In each experiment, all chicks were weighed, sorted by body weight into three body weight ranges (above average, average, and below average). Broiler chicks were selected from each of the body weight to minimize differences in initial cage body weight across treatments for a total of 350 chicks per experiment. Broilers were selected from each of the body weight ranges and placed in cages. Diets were randomly assigned to the cages with 10 replicates of 5 birds in each battery cage at a stocking density of 659 cm^2 per bird. Chicks were provided *ad libitum* access to experimental feed and water. Temperature was maintained according to breeder specifications based on the age of the

birds which ranged from 30° C at placement to 24° C at 11 days of age (Aviagen, 2018). Continuous lighting was provided from 0 to 3 days of age, then the lighting was adjusted to provide 20 hours of light and 4 hours of darkness from 3 to 11 days of age according to the commercial management guide (Ross 708 management guide). Health checks occurred twice daily and any mortality was removed from the cage, weighed and recorded.

DATA AND SAMPLE COLLECTION

Individual body weight and cage feed offered and refused were measured at the beginning and end of each experiment. Body weight gain and feed intake were calculated at the end of each experiment by the difference between final and initial body and feeder weight, respectively. Body weight gain and feed intake were used to calculate mortality corrected feed conversion ratio (FCRm) by adding the cage mortality body weight gain to cage bird body weight gain. At the end of each experiment, birds were euthanized using cervical dislocation and the breast muscle of all remaining birds was removed and weighed. The breast weight was expressed as a relative breast weight to body weight percentage.

STATISTICAL ANALYSIS

Digestible Lys and SAA requirement was estimated using both the linear and quadratic broken line models based on body weight gain, FCRm and relative breast weight. The Linear broken line model is based on an increasing or decreasing slope portion where the response is increasing with every increasing supplementation of a nutrient because the animal is below requirement for that specific nutrient, however, after the animal reaches the requirement, the result is a straight-line plateau without a slope. The point at which that plateau starts, or the line breaks is the maximum response and requirement estimate. Regression analysis was analyzed by

JMP non-linear model option of JMP 14 (SAS Institute Inc, Cary, NC) with formulated digestible Lys or SAA concentration as the independent variable. Digestible amino acid values were calculated using AminoDat Software (Version 5, 2016) by multiplying the digestibility coefficient of each ingredient by the amount of amino acid provided by that ingredient in the diet. When the LS means are provided with a ± sign, the second number presented is a pooled SEM.

RESULTS AND DISCUSSION

LYSINE REQUIREMENT

Chicks utilized in this experiment were from young broiler breeder hens and weighed approximately 37.5 g/bird when they reached our facilities after transportation from the hatchery. There were no differences in initial BW ($P > 0.05$) of broiler chicks in experiments 1 to 3 but were 1 or 2 days below breed expectations at start (Aviagen, 2019). This reduced body weight at the start is most likely due to small weights at hatch. In experiment 1, birds weighed 53 ± 0.22 g at the start of the experiment at 2-d. At 5 d, when experiment 2 started, birds weighed 103.0 ± 0.53 g. Finally, in experiment 3, 8-d old birds weighed 168 ± 1.7 g. Final BW for each experiment was 90 g on day 5 in experiment 1, 155 g on day 8 in experiment 2 and 260 g/bird on day 11 in experiment 3. With the low starting body weights, final body weights at the end of the three experiments were within 1 to 2 days of expected body weight in comparison to the performance objectives for Ross 708 broilers (Aviagen, 2019). This suggests that despite the low starting body weight, chicks were still growing as expected over the experimental period. The linear broken line model resulted in estimates of 1.22, 1.17, and 1.16% or 610, 772, and 1,283 of digestible lysine to maximize BWG from 2 to 5, 5 to 8, and 8 to 11 days of age, respectively

(Table 5). Requirements for FCR were estimated at 1.32, 1.21, and 1.14% or 660, 798, and 1,261 mg/d digestible lysine from 2 to 5, 5 to 8, and 8 to 11 days of age, respectively (Table 6). The linear broken line model did not result in estimates for maximum breast weight from 2 to 5 days of age, however the requirements from 5 to 8 and 8 to 11 days of age were 1.13 and 1.17 % or 751 and 1,294 mg/d of digestible lysine (Table 7). Requirement estimates for digestible Lys for BWG, and FCR increased with age on a mg/d basis, however when expressed on a percentage of diet basis, requirement estimates were reduced with chick age. These data validate the assumption of previous authors that digestible Lys requirements decrease with age when expressed on a dietary basis (Pope et al., 2002). The requirement estimates for breast weight increased from 5 to 8 d in comparison to 8 to 11 d of age which could indicate that the lysine digested earlier in life was used to drive structural development not maximization of breast weight. The linear-broken line Lys requirements of male Ross broiler chicks from 0 to 14 days of age were estimated at 1.28, 1.21, and 1.32 % Total Lys or 1.19, 1.12, and 1.23 % Digestible Lys for BWG, FCR and breast weight, respectively (Labadan et al., 2001). These results are similar to estimates for BWG in the current experiment. The Lys requirement estimates for FCR were similar, but slightly lower than the requirements reported in the current experiment. Breast weight requirements from 1 to 14 days were higher than the currently reported requirements which is consistent with the idea that older birds may have a higher Lys requirement for breast weight when compared to birds early in life. Previously, Cobb 500 broilers were raised in floor pens from 0 to 12 days of age resulting in digestible Lys requirements of 1.09 and 1.08% digestible Lys for BWG and FCR, respectively. These estimates were lower than those found in the current experiment or other reports for Ross strains (Cemin et al., 2014). This might suggest that broiler strains do have differences in digestible Lys requirements.

Results from the quadratic broken line analysis resulted in similar responses as the linear broken line model where the requirements of digestible Lys decreased from experiment 2 to 3 for BWG and FCR, respectively. No requirement was estimated for experiment 1 for all parameters for the quadratic broken line model due to a lack of quadratic response. Overall, the quadratic broken line resulted in higher estimates for all parameters compared to the linear broken line analysis. Additionally, the quadratic broken line analysis resulted in a better fit (R^2) compared to the linear broken line analysis. The Lys requirement of 0 to 7 and 0 to 14 d of age female Ross 708 and Cobb 500 broilers raised in floor pens were estimated using quadratic broken line models (Dozier and Payne, 2012). The digestible Lys requirement was estimated at 1.35 and 1.27 % for 0 to 7 and 0 to 14 d of age BWG for Ross broilers similar to the quadratic-broken line estimates reported in the current experiment. Female Cobb broilers had lower digestible Lys requirements of 1.27 and 1.18 % for BWG in comparison to the current experiment and previous data. This again supports the idea that there are differences in the efficiency of the utilization of Lys between strains. Similarly, the digestible Lys requirement for FCR was estimated at 1.27 % for 5 to 8 and 8 to 11 days but was not estimable at 2 to 5 days of age. As expected, these estimated from the quadratic broken line were higher than estimates from the linear broken line. Furthermore, these results were similar to the estimates reported in Cobb female broilers in a previous experiment from 0 to 14 days of age but lower than the estimated requirements for the Ross female broilers (Dozier and Payne, 2012).

SAA REQUIREMENT

Chicks utilized in this experiment were from young broiler breeder hens and weighed approximately 38.5 g/bird when they reached our facilities after transportation from the hatchery. There were no differences in initial BW ($P > 0.05$) of broiler chicks in experiments 1 to 3 but were between 1 and 2 days below breed expectations at start (Aviagen, 2019). This reduced body weight at the start is most likely due to lower weights at hatch. In experiment 4, birds weighed 58 ± 0.27 g at the start of the experiment at 2 d. At 5 d, when experiment 5 started, birds weighed 108 ± 0.53 g. Finally, in experiment 6, 8-day old birds weighed 165 ± 1.7 g. Final BW for each experiment was 105 ± 0.2 g on day 5 in experiment 4, 174 ± 1.4 g on day 8 in experiment 5 and 260 ± 2.2 g/bird on day 11 in experiment 6. With the low starting body weights, final body weights at the end of the three experiments were within 1 to 2 days of expected body weight in comparison to the performance objectives for Ross 708 broilers (Aviagen, 2019). This suggests that despite the low starting body weight, chicks were still growing as expected over the experimental period. The linear broken line model resulted in estimates of 0.82, 0.81, and 0.94% of SAA to maximize BWG from 2 to 5, 5 to 8, and 8 to 11 days of age, respectively (Table 8). Requirements for FCR were estimated at 0.82, 0.80, and 0.90 or 156.6, 248.5, and 303.8 mg/d for 2 to 5, 5 to 8, and 8 to 11 days of age, respectively (Table 9). The requirements for breast weight were estimated at 0.84, 0.82, and 0.98 or 160.4, 260.8, and 331.8 mg/d for 2 to 5, 5 to 8, and 8 to 11 days of age, respectively (Table 10). The SAA requirement in male Ross broilers were estimated at 0.91 % Total SAA or 0.82% digestible SAA for BWG and FCR using a linear broken line analysis (Skylan and Noy, 2003). Similarly, Garcia and Batal (2005) found a requirement in 2 experiments estimated at 0.82 and 0.84% for BWG and FCR using a linear broken line model in Cobb 500 male broilers from 0 to 7 days of age. These estimates are comparable to the results of this experiment for BWG from 2 to 5 and 5 to 8 days of age but slightly higher when FCR requirements are

considered. These results are somewhat surprising as it does not appear that SAA requirements for BWG have increased over this time period in the past 15 to 20 years of genetic selection. A requirement of 0.87% SAA was found using a quadratic polynomial model for optimizing BWG and FCR utilizing Cobb 500 broiler from 0 to 7 days of age (Goulart et al., 2011). A study utilizing the quadratic polynomial model found a comparable requirement of 0.89% in Ross 308 fast feathering male broilers from 0 to 21 days of age (Kalinowski et al., 2003). The quadratic polynomial model results in a higher modeled requirements compared to the linear or quadratic broken line used in our experiment which would explain the variation in estimates. The quadratic broken line estimates for SAA requirements expressed on a dietary basis increased when 2 to 5 d estimates were compared to 5 to 8 d estimates for BWG and FCR which was inconsistent with the linear broken line estimates. Although consistent with other reports, quadratic broken line estimates resulted in better overall fit for the data compared to the linear broken line (Sarsour et al., 2021).

In the current set of experiments, digestible SAA requirement estimates for BWG, FCRm, and relative breast weight increased with age on a mg/d basis, however when expressed on a percentage of the diet basis, the expected decrease was observed going from 2 to 5 days of age to 5 to 8 days of age. SAA requirements increased from 8 to 11 days of age. These increased SAA requirements from 8 to 11 days of age generally correspond with the feathering of chicks at this time and could be related to the role that sulfur amino acids play in feather production. Previous authors have reported a 0.05% increase in the SAA requirement of fast vs. slow feathering Ross broilers from 0 to 3 weeks of age (Kalinowski et al., 2003). Furthermore, Zeng and others (2015) reported that by increasing the SAA concentration in the diet from 0.55% to 0.81%, there was an increase in feather coverage in 28- and 35-day old pekin ducks. The current results indicate that

SAA requirements do not decrease over the starter period and at least for SAA the assumption that increased feed intake will reduce dietary amino acid needs over the starter period do not seem to hold true. This would indicate that before assumptions are made on the responses of specific amino acids requirements within a feeding phase, validation needs to be done due to the utilization of other amino acids for functions besides direct growth that might be driving or contributing to the requirement.

CONCLUSION AND APPLICATIONS

1. The linear broken line lysine requirement was estimated to be 1.22, 1.17 and 1.16% for BWG and 1.31, 1.21, and 1.14% for FCR from 2 to 5, 5 to 8, and 8 to 11 days of age, respectively.
2. Requirements for dietary lysine decreased with increased age and the increase in feed intake associated with older and larger chicks, validating the idea that within a feeding phase, dietary lysine requirement would decrease as chicks age.
3. The linear broken line SAA requirement was estimated at 0.82, 0.81 and 0.94% for BWG and 0.82, 0.80, and 0.90% for FCR from 2 to 5, 5 to 8, and 8 to 11 days of age, respectively.
4. Requirements for dietary SAA do not decrease with increased age within the starter period and do not follow the same responses as dietary lysine.
5. The idea of further refining feeding periods to more closely follow biological requirements within a feeding phase is still viable, but further validation of specific amino acid requirements should be completed due to potential non-growth related factors.

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Table 1. Composition of Lysine-deficient basal diet fed to broiler chicks from 2 to 5, 5 to 8, and 8 to 11 days of age, Experiments 1 to 3.[1]

Ingredient		Nutrient Profile	Formulated	Analyzed[2]
	(%)		——(%)——	
Corn	60.53	Crude protein	22.72	21.72
Soybean meal (47.5%)	22.49	ME (kcal/kg)	3030	---
Corn Gluten meal	7.45	Calcium	0.90	---
DDGS	5.00	Available P	0.45	---
Soy oil	0.20	Digestible Met	0.60	0.63 (0.59)
Sodium Chloride	0.20	Digestible Cys	0.28	0.35 (0.28)
Sodium Bicarbonate	0.10	Digestible SAA	0.88	0.98 (0.89)
L-Arginine	0.14	Digestible Lys	0.85	0.92 (0.81)
DL-Methionine	0.26	Digestible His	0.48	0.56 (0.51)
L-Lysine-HCl	0.00	Digestible Trp	0.19	0.24 (0.21)
L-Threonine	0.13	Digestible Thr	0.77	0.83 (0.71)
Limestone	0.95	Digestible Arg	1.24	1.22 (1.15)
Dicalcium Phosphate	1.82	Digestible Iso	0.79	0.93 (0.83)
Choline chloride (60%)	0.10			
Vitamin and Mineral premix[3]	0.63			

[1] L-Lysine was added at 0.10% increments to generate experimental diets that contained 0.85%, 0.95%, 1.05%, 1.15%, 1.25%, 1.35%, and 1.45% lysine

[2] Calculated amino acid values are reported as total dietary amino acid (digestible amino acid) and analyzed amino acid values are reported as total dietary amino acid. Analyzed total amino acid values were converted to digestible amino acid values using AminoDat Software (Version 5, 2016) by multiplying the digestibility coefficient of each ingredient by the amount of amino acid provided by that ingredient in the diet.

[3] Provided per kg of diet: vitamin A, 1,320,000 IU; vitamin D3, 440,000 ICU; vitamin E, 2860 IU; menadione, 176 mg; biotin, 6.6 mg; vitamin B12, 1.9 mg; choline, 71.5 g; niacin, 6.6 mg; pantothenic acid, 1.8 g; selenium, 40 mg; riboflavin, 880 mg; Cu, 4.4 g; Fe, 45 g; I, 135 mg; Mn, 44 g; Zn, 44 g; Co, 4.4 g.

Table 2. Formulated and analyzed lysine content of experimental starter diets fed to male broiler chicks over the 2 to 5, 5 to 8, and 8 to 11 d periods, experiment 1 to 3.[1]

Diet	Formulated		Analyzed	
	Total Lys	Digestible Lys	Total Lys	Digestible Lys[2]
	(%)			
Corn-SBM-CGM basal	0.96	0.85	0.92	0.81
Basal + 0.10% L-Lys	1.06	0.95	0.89	0.79
Basal + 0.20% L-Lys	1.16	1.05	1.01	0.89
Basal + 0.30% L-Lys	1.26	1.15	1.26	1.15
Basal + 0.40% L-Lys	1.36	1.25	1.33	1.18
Basal + 0.50% L-Lys	1.46	1.35	1.44	1.28
Basal + 0.60% L-Lys	1.56	1.45	1.47	1.31

[1] L-Lysine was added at 0.10% increments to generate experimental diets that contained 0.85%, 0.95%, 1.05%, 1.15%, 1.25%, 1.35%, and 1.45% lysine

[2] Calculated amino acid values are reported as total dietary amino acid (digestible amino acid) and analyzed amino acid values are reported as total dietary amino acid. Analyzed total amino acid values were converted to digestible amino acid values using AminoDat Software (Version 5, 2016) by multiplying the digestibility coefficient of each ingredient by the amount of amino acid provided by that ingredient in the diet.

Table 3. Composition of SAA-deficient basal diet fed to male broiler chicks from 2 to 5, 5 to 8, and 8 to 11 days of age, Experiments 4 to 6.[1]

Ingredient		Nutrient Profile	Formulated[2]	Analyzed
	(%)		(%)	
Corn	56.52	Crude protein	22.20	21.87
Soybean meal (48%)	35.66	ME (kcal/kg)	3030	---
Poultry byproduct meal	2.00	Calcium	0.90	---
Soy oil	1.85	Available P	0.45	---
Sodium Chloride	0.15	Digestible Met	0.34	0.37 (0.33)
Sodium Bicarbonate	0.27	Digestible Cys	0.29	0.34 (0.30)
DL-Methionine	0.02	Digestible SAA	0.63	0.71 (0.63)
L•Lysine-HCl	0.15	Digestible Lys	1.28	1.37 (1.25)
L Threonine	0.06	Digestible His	0.54	0.56 (0.51)
Limestone	0.88	Digestible Trp	0.24	0.28 (0.24)
Dicalcium phosphate	1.70	Digestible Thr	0.77	0.89 (0.79)
Choline chloride	0.10	Digestible Arg	1.39	1.50 (1.41)
Vitamin and Mineral premix[2]	0.63	Digestible Iso	0.85	0.91 (0.86)

[1] DL-Met was added to the basal diet at 0.07% increments to generate experimental diets that contained 0.63%, 0.70%, 0.77%, 0.83%, 0.90%, 0.97%, and 1.04% SAA.

[2] Calculated amino acid values are reported as total dietary amino acid (digestible amino acid) and analyzed amino acid values are reported as total dietary amino acid. Analyzed total amino acid values were converted to digestible amino acid values using AminoDat Software (Version 5, 2016) by multiplying the digestibility coefficient of each ingredient by the amount of amino acid provided by that ingredient in the diet.

[3] Provided per kg of diet: vitamin A, 1,320,000 IU; vitamin D3, 440,000 ICU; vitamin E, 2860 IU; menadione, 176 mg; biotin, 6.6 mg; vitamin B12, 1.9 mg; choline, 71.5 g; niacin, 6.6 mg; pantothenic acid, 1.8 g; selenium, 40 mg; riboflavin, 880 mg; Cu, 4.4 g; Fe, 45 g; I, 135 mg; Mn, 44 g; Zn, 44 g; Co, 4.4 g.

Table 4. Formulated and analyzed sulfur amino acid content of experimental starter diets fed to broiler chicks over the 2 to 5, 5 to 8, and 8 to 11 d periods, experiment 4 to 6.[1].

Diet	Formulated		Analyzed	
	Total SAA	Digestible SAA	Total SAA	Digestible SAA[2]
	(%)			
Corn-SBM-PBM basal	0.72	0.63	0.73	0.64
Basal + 0.07% DL-Met	0.79	0.70	0.85	0.76
Basal + 0.14% DL-Met	0.86	0.77	0.90	0.80
Basal + 0.21% DL-Met	0.93	0.83	0.94	0.84
Basal + 0.28% DL-Met	0.98	0.90	1.06	0.95
Basal + 0.35% DL-Met	1.05	0.97	1.13	1.01
Basal + 0.42% DL-Met	1.12	1.04	1.21	1.08

[1]DL-Met was added in 0.07% increments to generate experimental diets that contained 0.63%, 0.70%, 0.77%, 0.83%, 0.90%, 0.97%, and 1.04% SAA.

[2] Calculated amino acid values are reported as total dietary amino acid (digestible amino acid) and analyzed amino acid values are reported as total dietary amino acid. Analyzed total amino acid values were converted to digestible amino acid values using AminoDat Software (Version 5, 2016) by multiplying the digestibility coefficient of each ingredient by the amount of amino acid provided by that ingredient in the diet.

Table 5. Lysine requirements (mg/d and %) of Hubbard x Ross 708 broiler chicks estimated using linear and quadratic broken line models based on body weight gain (BWG) from 2 to 5, 5 to 8, and 8 to 11 days of age, Experiments 1 to 3[1].

BWG	Linear broken line Requirement		R^2	Quadratic broken line Requirement		R^2
(g)	(%)[2]	(mg/d)		(%)[3]	(mg/d)	
2 to 5 days	1.22	610	0.79	DNC[4]	DNC[4]	---
5 to 8 days	1.17	772	0.87	1.35	891	0.94
8 to 11 days	1.16	1,283	0.72	1.29	1,427	0.96
Labadan et al. (2001)						
0 to 14 d[5]	1.19[6]	---	---	---	---	---
Cemin et al. (2014)						
0 to 12 d[7]	1.09	---	0.87	1.17	---	0.88
Dozier and Payne (2012)						
0 to 7 d[8]	---	---	---	1.35[6]	---	---
0 to 7 d[9]	---	---	---	1.27[6]	---	---
0 to 14 d[8]	---	---	---	1.27[6]	---	---
0 to 14 d[9]	---	---	---	1.18[6]	---	---

[1] Requirements were estimated based on 10 cages of 5 broilers per cage which were fed 7 diets containing that contained 0.85%, 0.95%, 1.05%, 1.15%, 1.25%, 1.35%, and 1.45% lysine concentrations.
[2] 95% confidence intervals for linear broken line were 1.12 to 1.32, 1.12 to 1.22, and 1.13 to 1.23 for experiment 1, 2, and 3, respectively.
[3] 95% confidence intervals for quadratic broken line were 1.30 to 1.40, and 1.25 to 1.33 for experiment 2 and 3, respectively
[4] Did Not Converge - The response did not solve for the broken line model.
[5] Male Ross
[6] R^2 was not reported
[7] Male Cobb
[8] Female Ross
[9] Female Cobb

Table 6. Lysine requirements (mg/d and %) of Hubbard x Ross 708 broiler chickens estimated using linear and quadratic broken line models based on mortality corrected feed conversion ratio (FCRm) from 2 to 5, 5 to 8, and 8 to 11 d, Experiments 1 to 3[1].

FCRm (g:g)	Linear broken line			Quadratic broken line		
	Requirement		R^2	Requirement		R^2
	(%)[2]	(mg/d)		(%)[3]	(mg/d)	
2 to 5 days	1.32	600	0.72	DNC[4]	DNC[4]	---
5 to 8 days	1.21	772	0.84	1.27	810	0.94
8 to 11 days	1.14	1,283	0.81	1.27	1,429	0.92
Labadan et al. (2001)						
0 to 14 d[5]	1.12[6]	---	---	---	---	---
Cemin et al. (2014)						
0 to 12 d[7]	1.08	---	0.74	1.16	---	0.87
Dozier and Payne (2012)						
0 to 7 d[8]	---	---	---	1.38[6]	---	---
0 to 7 d[9]	---	---	---	DNC[4]	DNC[4]	---
0 to 14 d[8]	---	---	---	DNC[4]	DNC[4]	---
0 to 14 d[9]	---	---	---	1.26[6]	---	---

[1] Requirements were estimated based on 10 cages of 5 broilers per cage which were fed 7 diets containing that contained 0.85%, 0.95%, 1.05%, 1.15%, 1.25%, 1.35%, and 1.45% lysine concentrations.
[2] 95% confidence intervals for linear broken line were 1.21 to 1.43, 1.12 to 1.30, and 1.06 to 1.22 for experiment 1, 2, and 3, respectively.
[3] 95% confidence intervals for quadratic broken line were 1.12 to 1.42, and 1.18 to 1.36 for experiment 2 and 3, respectively.
[4] Did Not Converge - The response did not solve for the broken line model.
[5] Male Ross
[6] R^2 was not reported
[7] Male Cobb
[8] Female Ross
[9] Female Cobb

Table 7. Lysine requirements (mg/d and %) of Hubbard x Ross 708 broiler chickens estimated using linear and quadratic broken line models based on relative breast weight from 2 to 5, 5 to 8, and 8 to 11 d, Experiments 1 to 3[1].

Breast weight	Linear broken line			Quadratic broken line		
	Requirement		R^2	Requirement		R^2
	(%)[2]	(mg/d)		(%)[3]	(mg/d)	
2 to 5 days	DNC[4]	DNC[4]	---	DNC[4]	DNC[4]	---
5 to 8 days	1.13	751	0.86	1.27	844	0.95
8 to 11 days	1.17	772	0.69	1.44	950	0.99
Labadan et al. (2001)						
0 to 14 d[5]	1.23[6]	---	---	---	---	---

[1] Requirements were estimated based on 10 cages of 5 broilers per cage which were fed 7 diets containing that contained 0.85%, 0.95%, 1.05%, 1.15%, 1.25%, 1.35%, and 1.45% lysine concentrations.
[2] 95% confidence intervals for linear broken line were 1.08 to 1.18, and 1.11 to 1.23 for experiment 2 and 3, respectively.
[3] 95% confidence intervals for quadratic broken line were 1.21 to 1.33, and 1.34 to 1.54 for experiment 2 and 3, respectively
[4] Did Not Converge - The response did not solve for the broken line model.
[5] Male Ross
[6] R^2 was not reported

Table 8. Digestible sulfur amino acid requirements (mg/d and %) of Hubbard x Ross 708 broiler chickens estimated using linear and quadratic broken line models based on body weight gain (BWG) from 2 to 5, 5 to 8, and 8 to 11 d, Experiments 4 to 6[1].

Response criteria	Linear broken line			Quadratic broken line		
	Requirement		R^2	Requirement		R^2
BWG (g)	(%)[2]	(mg/d)		(%)[3]	(mg/d)	
2 to 5 days	0.82	156	0.80	0.85	173	0.95
5 to 8 days	0.81	253	0.86	0.91	288	0.94
8 to 11 days	0.94	321	0.74	0.98	406	0.96
Sklan and Noy (2003)						
0 to 7 d[4]	0.82	---	0.96	---	---	---
Garcia and Batal (2005)						
0 to 7 d[5]	0.82[6]	---	---	---	---	---
Goulart et al. (2011)						
0 to 7 d[5]	---	---	---	0.87[6]	183	---
Kalinowski et al. (2003)						
0 to 21 d[4]	---	---	---	0.89[6]	---	---

[1] Requirements were estimated based on 10 cages of 5 broilers per cage which were fed 7 diets containing that contained 0.63%, 0.70%, 0.77%, 0.83%, 0.90%, 0.97%, and 1.04% SAA concentrations.
[2] 95% confidence intervals for linear broken line were 0.79 to 0.85, 0.79 to 0.83, and 0.88 to 1.00 for experiment 4, 5, and 6, respectively.
[3] 95% confidence intervals for quadratic broken line were 0.78 to 0.86, 0.86 to 0.96, and 0.90 to 1.06 for experiment 4, 5, and 6, respectively.
[4] Male Ross 308
[5] Male Cobb 500
[6] R^2 was not reported

Table 9. Digestible sulfur amino acid requirements (mg/d and %) of Hubbard x Ross 708 broiler chickens estimated using linear and quadratic broken line models based on mortality corrected feed conversion ratio (FCRm) from 2 to 5, 5 to 8, and 8 to 11 d, Experiments 4 to 6[1].

FCR (g:g)	Linear broken line			Quadratic broken line		
	Requirement		R^2	Requirement		R^2
	(%)[2]	(mg/d)		(%)[3]	(mg/d)	
2 to 5 days	0.82	156	0.74	0.87	170	0.94
5 to 8 days	0.80	248	0.72	0.95	279	0.94
8 to 11 days	0.90	303	0.71	0.96	338	0.92
Sklan and Noy (2003)						
0 to 7 d[4]	0.82	---	0.92	---	---	---
Garcia and Batal (2005)						
0 to 7 d[5]	0.84[6]	---	---	---	---	---
Goulart et al. (2011)						
0 to 7 d[5]	---	---	---	0.87[6]	183	---
Kalinowski et al. (2003)						
0 to 21 d[4]	---	---	---	0.89[6]	---	---

[1] Requirements were estimated based on 10 cages of 5 broilers per cage which were fed 7 diets containing that contained 0.63%, 0.70%, 0.77%, 0.83%, 0.90%, 0.97%, and 1.04% SAA concentrations.
[2] 95% confidence intervals for linear broken line were 0.78 to 0.86, 0.76 to 0.84, and 0.82 to 0.98 for experiment 4, 5, and 6, respectively.
[3] 95% confidence intervals for quadratic broken line were 0.82 to 0.92, 0.90 to 1.00, and 0.88 to 1.04 for experiment 4, 5, and 6, respectively.
[4] Male Ross 308
[5] Male Cobb 500
[6] R^2 was not reported

Table 10. Digestible sulfur amino acid requirements (mg/d and %) of Hubbard x Ross 708 broiler chickens estimated using linear and quadratic broken line models based on relative breast weight from 2 to 5, 5 to 8, and 8 to 11 d, Experiments 4 to 6.[1,2]

Response criteria	Linear broken line			Quadratic broken line		
	Requirement[2]		R^2	Requirement[3]		R^2
Breast weight (%)	(%)	(mg/d)		(%)	(mg/d)	
2 to 5 days	0.84	160	0.74	0.97	203	0.94
5 to 8 days	0.82	260	0.79	0.95	312	0.95
8 to 11 days	0.98	331	0.81	1.17	503	0.99

[1] Requirements were estimated based on 10 cages of 5 broilers per cage which were fed 7 diets containing that contained 0.63%, 0.70%, 0.77%, 0.83%, 0.90%, 0.97%, and 1.04% SAA concentrations.
[2] 95% confidence intervals for linear broken line were 0.78 to 0.90, 0.78 to 0.86, and 0.90 to 1.06 for experiment 4, 5, and 6, respectively.

[3] 95% confidence intervals for quadratic broken line were 0.91 to 1.03, 0.90 to 1.00, and 1.03 to 1.31 for experiment 4, 5, and 6, respectively.
[4] No previous breast weight data have been reported over this timeframe of broiler development.

Scientific section: Management and Production

CHAPTER 5: EFFECTS OF SULFUR AMINO ACID SUPPLEMENTATION ON BROILER CHICKENS EXPOSED TO ACUTE AND CHRONIC CYCLIC HEAT STRESS

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ABSTRACT

Chronic heat stress can result in oxidative damage from increased reactive oxygen species. One proposed method to alleviate these chronic effects of HS is the supplementation of sulfur amino acids (SAA) which can be metabolized to produce glutathione, an important antioxidant. Therefore, the objective of this experiment was to determine the effects of dietary SAA content on broiler chickens exposed to HS from 28 to 35 d on broiler performance, body temperature, intestinal permeability, and oxidative status. Four experimental treatments were arranged as a 2 x 2 factorial consisting of HS (6 h at 33.3 °C followed by 18 h at 27.8 °C from 28 to 35 d of age) and Thermoneutral (TN- 22.2 °C continuously from 28 to 35 d) and 2 dietary concentrations of SAA formulated at 100% (0.95, 0.87, and 0.80% for starter, grower, and finisher diets) or 130% SAA (1.24, 1.13, and 1.04% for starter, grower, and finisher diets). A total of 648-day-old, male Ross 708 chicks were placed in 36 pens with 18 chicks/pen and 9 replicates per treatment. Data were analyzed as a 2 x 2 factorial in JMP 14 ($P \leq 0.05$). No interaction effects were observed on broiler live performance ($P > 0.05$). As expected, HS reduced BWG by 92 g and increased FCR by 11 points from 28 to 35 d of age compared to TN, respectively ($P \leq 0.05$). The supplementation of SAA had no effect on live performance ($P > 0.05$). Cloacal temperatures were increased by 1.7, 1.4 and 1.2 °C with HS at 28, 31, and 35 d compared to TN, respectively ($P \leq 0.05$) and dietary SAA did not alter cloacal temperatures. At 28 d of age, supplementation of SAA to birds exposed to HS interacted as serum FITC-dextran (an indicator of intestinal permeability) was reduced to that of the TN group ($P \leq 0.05$). The interaction was lost at 31 d, but HS still increased intestinal permeability ($P \leq 0.05$). By 35 d, broilers were able to adapt to the HS conditions and intestinal permeability was unaffected ($P > 0.05$). Potential oxidative damage was reduced by increased SAA supplementation as indicated by an improvement in the reduced glutathione to oxidized glutathione ratio of 5 and 45 % at 28

(P = 0.08) and 35 d (P ≤ 0.05). These data suggest that intestinal permeability is compromised acutely to at least three days of heat exposure before the bird can adjust. However, the oxidative damage is more chronic building over the entire 7 d HS period and increased dietary SAA might have some protective effect on both intestinal permeability and oxidative stress responses to HS.

Key words: Sulfur amino acids, broilers, heat stress, intestinal permeability, oxidative stress

INTRODUCTION

Genetic selection has increased both the growth rate and efficiency of broilers over the last 60 years contributing to over 400% improvement in growth rates with a 50% improvement in feed and water efficiency (Zuidhof et al., 2014). As a result of this increased growth rate, there is an associated increase in metabolic heat production with these faster-growing broilers which will reduce the bird's ability to mitigate heat stress when they are subjected to elevated temperatures (Nascimento et al., 2017). The first mechanism of heat mitigation includes posture changes with wings to decrease feather coverage and insulation, as well as digging in the litter to find a cooler surface for heat loss by conduction (Mack et al., 2013). Once passive or minimally active methods are exhausted, birds will use evaporative heat loss through panting to cool core body temperatures (Mack et al., 2013).

A previous heat stress experiment using a pair feeding strategy, indicated that approximately 63% of the growth depression in heat stressed chickens can be attributed to the reduced feed intake with HS (Dale and Fuller, 1979). Although the majority of body weight gain loss is attributed to reduced feed intake, a third of the weight loss could be due to physiological responses. One of the physiological responses that can reduce growth rate during HS is intestinal permeability. Heat stress can increase intestinal permeability by impairing the tight junction proteins that form a barrier between enterocytes (Quinteiro-Filho et al., 2010). The breakdown of

tight junction proteins can result in leaking of bacterial metabolites or bacteria into circulation resulting in reduced performance or potential for disease (Wu et al., 2018). Tight junction leaking has been hypothesized to occur through two different mechanisms. First is the increase of blood flow being directed to peripheral tissue to increase heat loss thus reducing the blood flow to the gastrointestinal tract during HS, which can result in reduction in nutrients and oxygen to the intestinal tract causing damage to the different parts of the intestine (Lambert, 2009). Secondly, a down regulation of tight junction protein expression and is correlated with an increase in intestinal permeability which could be related to hypoxic effects of HS (Tabler et al., 2020).

In addition to increased intestinal permeability, HS can also result in over production of ROS. Reactive oxygen species are natural byproducts that result from cellular oxidative metabolism in the mitochondria (Turrens, 2003). The increase in energy demand within the body to combat heat stress and return to homeostasis can increase production of ROS (Yang et al., 2010). Heat stress can cause damage to the mitochondria resulting in dysfunction of the electron transport chain and further increases ROS production (Mujahid et al., 2005). Additionally, ROS can further oxidize proteins and lipids during HS resulting in the production of more radicals that can further damage cells (Benzie, 1996). Normal ROS levels are maintained in cells with the help of antioxidant enzymes produced endogenously within the body such as superoxide dismutase, catalase, and glutathione peroxidase (Yang et al., 2010). Glutathione plays a direct role in mitigation of peroxides by transferring 2 hydrogen atoms resulting in the formation of a stable water molecule. Oxidized glutathione can be recycled back to reduced glutathione by the donation of 2 hydrogen atoms from Nicotinamide adenine dinucleotide phosphate (NADPH) catalyzed by the enzyme glutathione reductase. During oxidative stress, there is a rapid and

substantial decline in the glutathione level in the body, so finding strategies to increase the glutathione will potentially improve the antioxidant status within the body maintaining that balance of ROS and antioxidants (Willemsen et al., 2011). Reduced glutathione to oxidized glutathione ratio (rGSH:GSSG) is often used to determine the redox status since under normal conditions most of the glutathione would be in the rGSH form (Owen and Butterfield, 2010).Glutathione is synthesized in the liver of birds from the amino acid cysteine which can be synthesized from the sulfur amino acid methionine.

The SAA, methionine and cysteine, are essential to poultry diets as they are used for muscle development and growth (Garcia and Batal, 2005). However, during HS, additional supplementation of SAA might reduce the effects of elevated temperatures on birds. Previous research in piglets has shown that supplementation of methionine can result in a reduction in intestinal permeability due to the increase in expression of tight junction proteins (Chen et al., 2014). One of the mechanisms for SAA improving intestinal permeability is through the reduction in ROS species which can damage cells and DNA within the body. The other mechanism is through the production of polyamines which methionine acts as the primary donor for their synthesis. Polyamines are important in protecting the intestine as well as the tight junction proteins and the result of polyamine depletion would result in the disruption of the barrier function (Guo et al., 2005). The effect of SAA on intestinal permeability has not been investigated in poultry under heat stress; however, in an in-vitro experiment Caco-2 cells were challenged with hydrogen peroxide resulting in an impairment of epithelial barrier function that was ameliorated with supplemental methionine (Martin-Venegas et al., 2013). Previous research has also reported that supplementation of SAA to birds under HS resulted in reduced ROS

production, and an increase in antioxidant activity in quail compared to TN (Del Vesco et al., 2014). Therefore, the objective of the current experiment was to investigate the effects of SAA supplementation on broiler chickens exposed to a cyclic heat stress on broiler performance, cloacal temperature, panting, intestinal permeability, and oxidative stress**.**

MATERIALS AND METHODS

DIET FORMULATION AND PRODUCTION

The corn, soybean meal, DDGS and poultry byproduct meal used in this experiment were analyzed for amino acid concentrations by wet chemistry (AOAC method 982.03) prior to dietary formulation to generate a control and control plus 30% SAA diets. Broiler diets were formulated using a phase feeding approach including starter (0 to 11 d), grower (11 to 21 d), and finisher (21 to 35 d) (Table 1) to meet nutrient recommendations (Aviagen, 2018). A basal diet approach was used where common ingredients were mixed for each dietary phase before being equally split to generate two experimental diets. The diets formulated to 100% of the SAA requirement contained 0.95, 0.87, and 0.80 % while the diets formulated to 130% of the requirement contained 1.24, 1.13, and 1.04 % SAA for starter, grower, and finisher diets, respectively. Experimental diets were analyzed for amino acid content using wet chemistry (AOAC method 982.03).

EXPERIMENT DESIGN AND BROILER MANAGEMENT

All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech (Blacksburg, VA). Treatments were arranged as a 2 x 2 factorial with temperature: Elevated (HS-6 h at 33.3 °C followed by 18 h at 27.8 °C from 28 to 35 d of

age) and Thermoneutral (TN- 22.2 °C continuously from 28 to 35 d) and 2 dietary concentrations of SAA (0.95, 0.87, and 0.80 % or 1.24, 1.13, and 1.04 % for starter, grower, and finisher diets, respectively) as the two factors. In total, 648 Ross 708 broiler chicks were allotted to the 4 treatments with 9 replicate pens of 18 broilers in two different rooms. Broilers were provided *ad libitum* access to experimental feed and water. Temperature was maintained according to breeder specifications based on the bird age starting at 30 °C at placement to 22 °C at 28 days of age (Aviagen, 2018). At 28 days of age, the TN room maintained 22 °C continuously from 28 to 35 d. The HS room was adjusted to 33.3 °C for 6 hours followed by 18 hours at 27.8 °C and continued in this cyclic fashion daily from 28 to 35 d of age. Continuous lighting was provided from 0 to 3 days of age, followed by 20 hours of light and 4 hours of darkness from 3 to 35 days of age. Health checks occurred at least twice daily when mortality was noted it was removed from the pen, weighed, and recorded.

BROILER PERFORMANCE AND BODY COMPOSITION

Birds were weighed individual on day 0, 11, 21, 28, and 35 when diet phases changed or HS was initiated and body weight gain was determined over the 0 to 28 d pre-HS period, the 28 to 35 d HS period and the entire 0 to 35 d period as the difference between final and initial body weights. Feed offered and refused was determined on the same schedule of 0, 11, 21, 28 and 35 days and feed intake calculated as the difference between feed offered and refused for the 0 to 28 d pre-HS period, the 28 to 35 d HS period and the entire 0 to 35 d period. Body weight gain and feed intake by pen along with mortality weights were used to calculate mortality corrected feed conversion ratio (FCRm) by adding the pen mortality body weight gain to pen bird body weight gain. At 35 days of age, 5 broilers per pen were randomly selected, euthanized and defeathered. Dual X-ray absorptiometry (DXA) with a Lunar Prodigy machine (GE Lunar, GE Healthcare,

Waukesha, WI) was utilized to measure fat and protein content of carcasses. Extra chicks that were not selected for the experiment at day 0 were euthanized and scanned for DXA as baseline measures of chick body composition. Protein and fat accretion was calculated by the difference in protein and fat from 0 to 35 days by day.

CLOACAL TEMPERATURE AND PANTING

Cloacal temperature was measured at 28 and 35 days of age from 5 randomly selected broilers per pen. Cloacal temperatures were measured by inserting a thermometer (DeltaTrak MDL11064) exactly 2 cm into the cloaca of each broiler. Individual cloacal temperatures were used as subsamples and averaged to determine a pen value for statistical analysis. Panting observations were performed by the same observer at 32 and 33 days of age on all broilers within the pen 2 hours after heat stress was initiated and 1 hour after heat stress was concluded. The observation was done on a scan sampling basis where the frequency of birds panting was observed. Panting was defined as an open beak with an abnormally rapid respiration rate. Non-panting was defined as a closed beak with normal respiration rate (Mack et al., 2013).

OXIDATIVE STRESS

One bird per pen was randomly selected on 28 and 35 days of age. These birds were euthanized 2 hours after the initiation of HS and a liver sample collected and flash frozen in liquid nitrogen. A 0.3 g frozen liver sample was thawed, homogenized in 3 ml of 0.9% PBS using a bench top homogenizer (Tekmar company, Ohio, USA). Total glutathione (TGSH), Oxidized glutathione (GSSG) and reduced glutathione (rGSH) were measured using an enzymatic recycling method (Cayman Chemical Company, Ann Arbor, MI). The sample was then deproteinated, and then the GSH in the sample reacted with DTNB (5,5'-dithio-bis-2-

nitrobenzoic acid) to produce the yellow colored 5-thio-2- nitrobenzoic acid (TNB). The mixed disulfide, GSTNB, was reduced by glutathione reductase to recycle the GSH and produce more TNB. The amount of TNB production was quantified colorimetrically using the Infinite M200 Pro (Tecan, Morrisville, NC) set at 410 nm. The TNB concentration was directly proportional to the concentration of TGSH in the sample. Addition of glutathione peroxidase caused GSH to be oxidized to GSSG. Quantification of GSSG, was accomplished by first adding 2- vinylpyridine to GSH and the same methods as above were used to quantify GSSG. One molecule of GSSG in the body gets converted to 2 rGSH so there is double the amount of rGSH compared to GSSG. Thus, the rGSH levels were calculated by substracting twice the GSSG concentration from the TGSH concentration. The rGSH to GSSG ratio were also calculated to determine the redox status since under normal conditions most of the glutathione would be in the rGSH form (Owen and Butterfield, 2010).

INTESTINAL PERMEABILITY

Intestinal permeability was estimated using a FITC-dextran (FITC-d) model (Baxter et al., 2017). This model is based on the fact that a large molecule dextran is not easily absorbed into the body, especially through the enterocyte itself. When intestinal structure is compromised, dextran can leak into circulation so quantifying serum dextran using a dosed and labeled dextran molecule can be used as a proxy for intestinal permeability. On 28, 31, and 35 days of age, one broiler per pen was randomly selected and orally gavaged with 8.32 mg/kg of FITC-d dissolved in double distilled water 1 h before blood sample collection. Blood was collected from the brachial vein in serum tubes and allow to clot for 4 h at room temperature under dark conditions. Serum was isolated from the blood by centrifuging tubes at 2000 × g for 10 min at 4°C. Serum was then removed and diluted 1:5 in sterile 0.9% saline to a total volume of 100 µl in 96 well flat

bottom black plate. The serum was analyzed for FITC-d at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using multi-mode plate reader (Infinite M200 Pro, Tecan, Morrisville, NC). Serum fluorescent concentrations were then determined using a standard curve of FITC-d sera generated by direct addition of FITC-d into sera of control chickens from the same experiment that had not received FITC-d.

STATISTICAL ANALYSIS

Data were analyzed as a 2 x 2 factorial with HS and SAA as the main effects in JMP 14. The normal distribution of the data was verified by assessing the normal quantile plots in JMP 14. Student's t-test was used to separate significant least squares means with the probability set at $P \leq 0.05$. Treatments were randomly allotted among blocks within rows that were used as the random variable in the analyses. All data were analyzed based on the pen level experimental unit. The BWG at 0 to 28 days was added to our statistical model as a covariate for 28 to 35 BWG analyses.

RESULTS AND DISCUSSION

BROILER PERFORMANCE AND BODY COMPOSITION

The analyzed SAA were similar to formulated values in the grower and finisher diet phases but were lower than expected in the methionine supplemented diet within the starter phase (Table 1). Nevertheless, there was still an 0.07% increase in SAA with the supplemented group compared to non-supplemented group. There was no difference in initial BW ($P > 0.05$) at placement and chicks averaged 43.5 g/bird. No interactions were observed between HS and dietary SAA for live performance or body composition ($P > 0.05$; Table 2 and 3). Dietary SAA had no effect on the performance of broilers regardless of heat treatment. Previous experiments

have concluded that when diets are formulated to be in excess of SAA requirement for maximum growth in TN conditions, there is no additional improvement in growth observed of broilers subjected to HS (Willemsen et al., 2011, Liu et al., 2019, Zeitz et al., 2020). From hatch to 28 d of age, (the period before HS) birds raised in the HS room resulted in a 40 g decrease in BWG and a 6-point worsening of FCRm ($P \leq 0.05$). This result was unexpected, but these differences were used as a covariate for analysis of the 28 to 35 d data to minimize any carryover effects. As expected, broilers that were subjected to elevated temperature had reduced BWG and worsened FCR ($P \leq 0.01$) from 28 to 35 d and 0 to 35 d compared to the broiler raised under TN conditions. This is consistent with previous research that demonstrated that HS resulted in a reduction in overall growth and feed efficiency (Deeb and Cahaner, 2002; Niu et al., 2009; Imik et al., 2012; Sohail et al., 2012). As expected with the reduced BW, HS resulted in reduced protein and fat accretion from 0 to 35 days of age compared to broilers that were raised under TN conditions ($P \leq 0.01$). Arbor Acre broiler chickens subjected to a cyclic HS (23.9 to 35 °C) from day 28 to 49, observed a decrease in breast weight and abdominal fat in the HS group compared to the TN broilers at 49 days of age (Smith, 1993). Taken together these data suggest that HS reduces body weight overall and is not selective for either a reduction in lean tissue or fatty tissue at the expense of the other. As with broiler performance, lean or fatty tissue accretion was not altered by SAA supplementation ($P > 0.05$).

CLOACAL TEMPERATURE AND PANTING

Heat stress was confirmed by measuring broiler cloacal temperatures 2 hours after the initiation of HS on 28, 31, and 35 d of age. No interactions were observed between HS and SAA on cloacal temperature or panting ($P > 0.05$; Table 4). Broilers that were subjected to HS had increased cloacal temperatures 2 hours after the initiation of HS at 28, 31 and 35 days of age

compared to broilers under TN temperatures ($P \leq 0.01$). The incidence rate of panting in broilers that were subjected to HS was increased to 96 ± 2.4% 2 hours after HS exposure compared to 6.66 ± 2.2% broilers that were under TN conditions on both 32 and 33 days ($P \leq 0.01$). The panting effect was still observed one hour after the conclusion of HS, but to a lesser extent at 31% incidence ($P \leq 0.01$). Supplementation of diets with SAA reduced panting 2 hours after the initiation of HS on 32 d of age ($P \leq 0.05$); however, this effect was inconsistent and not shown on 33 d or after the conclusion of HS on either day ($P > 0.05$). There is no clear mechanism of how SAA can reduce panting, but Cobb 500 male broilers subjected to a moderate continuous HS at 27.4°C found reduced respiration rate with supplementation of methionine to broilers under HS compared to broilers with no additional supplementation at 4 weeks of age but no effect at 3 or 5 weeks of age (Zeitz et al., 2020). Previous experiments have reported consistent HS responses on body temperature and panting (Felver-Gant et al., 2012; Mack et al., 2013; Zeitz et al., 2020). However, consistent reduction have not been reported with SAA under HS conditions but could possible have to do with the reduction in oxidative stress due to the activity of glutathione.

OXIDATIVE STRESS

A trend ($P = 0.08$) was observed for an interaction during the acute phase of HS at 28 days between environmental temperature and SAA supplementation on the rGSH:GSSG ratio (Figure 1a; Table 5). Liver tissue of broilers subjected to HS had a reduced rGSH:GSSG ratio compared to livers of broiler under TN temperature indicating a reduction in redox potential. After just 2 hours of HS, supplementation of SAA to broilers partially improved the redox status on day 28 (Figure 1a; $P = 0.08$). After birds were exposed to cyclic HS for 7 days, the effects of increased dietary SAA were more apparent in reducing the ratio of rGSH:GSSG in broiler

subjected to HS compared to TN exposed broilers (Figure 1b; $P \leq 0.05$). These results are consistent with a previous report with male Ross broilers subjected to a continuous HS at 32 °C from 2 to 6 weeks of age. They reported a reduction in redox capacity with the HS compared to TN while showing mitigation of this reduction with additional methionine supplementation (Willemson et al., 2011). Cobb 500 broilers subjected to acute HS at 38 °C for 24 hours at 21 days of age reported an increase in glutathione peroxidase activity with supplementing excessive methionine during heat stress (Del Vesco et al., 2015). Glutathione peroxidase catalyzes the reduction of free radical damage to harmless molecules. This implies that SAA not only reduce oxidative stress but also improve the activity of key enzymes needed for this process. Male Cobb 500 broilers subjected to a moderate continuous HS at 27.4°C from 3 to 5 weeks of age found no effect on the rGSH:GSSG ratio but found that the additional supplementation of SAA increased the hepatic concentration of both rGSH and GSSG (Zeitz et al., 2020). These data suggest that higher temperatures are required to generate an oxidative response in broilers, but supplemental SAA are an important precursor to maintain oxidative balance.

INTESTINAL PERMEABILITY

Two hours after first initiation of HS on 28 d of age, there was an interaction observed between temperature and SAA supplementation (Figure 1c; $P \leq 0.05$). Heat stress without additional dietary SAA resulted in an increase of serum FITC-d compared to TN birds that was mitigated by the higher dietary SAA in the HS group. This interaction suggests a more permeable intestine generated by the HS that was ameliorated by the increased supplementation of SAA. Broilers subjected to HS had higher serum FITC-d concentrations compared to TN group at 31 days of age regardless of SAA supplementation (Table 6; $P \leq 0.05$) indicating compromised intestinal structure and a “leaky” gut. After the broilers had been subjected to the

cyclic HS for 7 d, the serum FITC-d concentrations similar to those of the TN groups ($P > 0.05$). These results are consistent with a previous experiment investigating the effects of an acute 36 °C heat stress for 2 hours which resulted in an increase in serum FITC-d concentration in several genetic lines of broilers compared to TN (Tabler et al., 2020). This increase in FITC-d was related to downregulation of several tight junction proteins in both the jejunum and ileum. A continuous 35°C HS treatment was provided to 21 to 42 d of age Cobb 500 broilers which resulted in serum FITC-d concentrations increasing with the HS at both 35 and 42 days of age (Ruff et al., 2020). The disruption of tight junction proteins has been associated with secretion of pro-inflammatory cytokines into the intestinal tract which could cause reduced performance and efficiency (Bailey et al., 2011). Oxidative stress and the increase in free radicals have also been reported to disrupt these tight junctions in the intestine and increase intestinal permeability (Sanders et al., 2005). This could partially explain the weight gain and efficiency losses when broilers are subjected to HS.

In conclusion, SAA supplemented at 130% of the requirement did not improve any of the performance parameters of broilers exposed to a cyclic heat stress. However, SAA supplementation was able to improve the antioxidant function of broilers exposed to HS as demonstrated by the increase of the ratio of rGSH:GSSG through an increase in the production of the antioxidant glutathione partially during the acute response to HS and significantly after chronic HS exposure. The supplementation of SAA reduced the intestinal permeability of broilers during the acute phase which is associated with the leaking of pathogens and metabolites to circulation. In this experiment, the effects of HS on oxidative stress were more prominent over time but the effects on intestinal permeability were more pronounced with first acute exposure and diminished overtime.

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Table 1. Formulation and nutrient profile of experimental diets for starter (0-11 d), grower (11-21 d) and finisher (21-35 d) diets fed to Ross 708 broilers and exposed to elevated environmental temperatures from 28 to 35 d.[1]

Ingredient	Starter	Grower	Finisher
	(%)		
Corn	58.28	60.39	64.98
Soybean meal	33.39	29.66	23.59
Poultry byproduct meal	2.00	5.00	6.00
Soy oil	0.68	1.82	2.63
Sodium Chloride	0.18	0.17	0.16
Sodium-bicarbonate	0.20	0.20	0.20
DL-Methionine	0.33	0.27	0.24
L-Lysine	0.22	0.13	0.18
L-Threonine	0.08	0.03	0.04
Limestone	0.92	0.86	0.80
Dicalcium phosphate	0.97	0.86	0.56
Phytase[2]	0.01	0.01	0.01
Choline chloride	0.10	0.10	0.10
V & M premix[3]	0.63	0.52	0.52

Nutrient	Starter		Grower		Finisher	
	100%	130%	100%	130%	100%	130%
	(%)					
Crude protein	22.8	23.0	21.6	21.8	19.7	19.9
ME, kcal/kg	3000	3000	3100	3100	3200	3200
Calcium	0.80	0.80	0.77	0.77	0.69	0.69
Nonphytate P	0.35	0.35	0.34	0.34	0.30	0.30
Fat	3.90	3.87	5.16	5.14	6.17	6.15
Met	**0.62 (0.62)**	**0.91 (0.69)**	**0.55 (0.56)**	**0.81 (0.77)**	**0.51 (0.47)**	**0.75 (0.65)**
Cys	**0.33 (0.43)**	**0.33 (0.43)**	**0.32 (0.41)**	**0.32 (0.41)**	**0.29 (0.34)**	**0.29 (0.34)**
Met+Cys	**0.95 (1.05)**	**1.24 (1.12)**	**0.87 (0.97)**	**1.13 (1.18)**	**0.80 (0.81)**	**1.04 (0.99)**
Lys	1.28 (1.25)	1.28 (1.25)	1.15 (1.13)	1.15 (1.13)	1.06 (1.13)	1.06 (1.13)
His	0.56	0.56	0.53	0.53	0.48	0.48
Trp	0.23	0.23	0.22	0.22	0.19	0.19
Thr	0.86 (0.81)	0.86 (0.81)	0.77 (0.72)	0.77 (0.72)	0.71 (0.72)	0.71 (0.72)
Arg	1.39	1.39	1.31	1.31	1.17	1.17
Iso	0.85	0.85	0.80	0.80	0.71	0.71
Val	0.94	0.94	0.90	0.90	0.81	0.81

[1]Calculated amino acid values are reported as total dietary amino acid (digestible amino acid) and analyzed amino acid values are reported as total dietary amino acid.
[2]Quantum blue (500 FTU/kg) was formulated to provide 0.10% of calcium and nonphytate phosphorus
[3] Vitamin and mineral premix Provided per kg of diet: vitamin A, 4403 IU; vitamin D3, 1457 ICU; vitamin E, 1.10 IU; menadione, 0.77 mg; vitamin B12, 4.40 µg; choline, 254.79 mg; niacin, 13.21 mg; pantothenic acid, 4.05 mg; riboflavin, 2.75 mg; Cu, 2.70 mg; Fe, 33.75 mg; I, 0.67 mg; Mn, 42.90 mg; Zn, 32.50 mg; Co, 0.17 mg.

Table 2. The effect of SAA supplementation on Body weight gain and mortality corrected feed conversion ratio (FCRm) on broilers over the 0 to 35 d period when exposed to heat stress from 28 to 35 d.[1]

Temperature[2]	Diet[3]	Body weight Gain			FCRm (g:g)		
		0 to 28	28 to 35	0 to 35	0 to 28	28 to 35	0 to 35
		(g)			(g feed/g gain)		
TN		1,388[a]	741[a]	2,129[a]	1.561[b]	1.762[b]	1.602[b]
HS		1,348[b]	649[b]	1,997[b]	1.620[a]	1.866[a]	1.661[a]
Pooled SEM		13	17	23	0.032	0.034	0.022
	Normal DSAA	1,386	689	2,075	1.580	1.841	1.651
	130% DSAA	1,350	701	2,051	1.609	1.782	1.623
Pooled SEM		13	17	23	0.032	0.034	0.022
P Value							
Temperature		**0.05**	**≤ 0.01**	**≤ 0.01**	**0.02**	**≤ 0.01**	**≤ 0.01**
Diet		0.10	0.72	0.16	0.23	0.31	0.41
Temperature x Diet		0.20	0.88	0.11	0.79	0.26	0.55

[1] Values are means from 9 replicate pens from each interaction or 18 per main effect.

[2] TN = continuous 22 to 24°C; HS received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.

[a-b] Values in a column without common superscript letter are different ($P \leq 0.05$)

Table 3. The effect of SAA supplementation on the overall lean and fat tissue accretion from 0 to 35 days of age of broiler chickens exposed to heat stress from 28 to 35 d in both experiments.[1]

Temperature[2]	Diet[3]	Protein (g/day)	Fat (g/day)
TN		56.6[a]	9.8[a]
HS		52.0[b]	8.7[b]
Pooled SEM		1.31	0.12
	Normal DSAA	53.8	9.3
	130% DSAA	54.8	9.1
Pooled SEM		1.31	0.12
P Value			
Temperature		**0.02**	**≤ 0.01**
Diet		0.57	0.31
Temperature x Diet		0.57	0.59

[1] Values are means from five birds per pen from 9 replicate pens from each interaction or 18 per main effect.

[2] TN = continuous 22 to 24°C; HS received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.

[a-b] Values in a column without common superscript letter are different ($P \leq 0.05$)

Table 4. The effect of SAA supplementation on cloacal temperature and panting (measured 2 h after daily heat exposure on d 28, 31 and 35 and 1 h after end of HS for panting) broilers exposed to heat stress from 28 to 35 days of age

Temperature program[2]	Diet[3]	Cloacal temperature			Panting			
		28	31	35	32d	32d 1hr after hs	33d	33d 1hr after hs
		°C			%			
TN		41.9[b]	41.9[b]	41.8[b]	6.66[b]	3.52[b]	2.13[b]	3.62[b]
HS		43.7[a]	43.3[a]	43.0[a]	95.53[a]	31.60[a]	91.57[a]	38.46[a]
Pooled SEM		0.07	0.06	0.04	2.51	2.68	1.72	2.72
	Normal DSAA	42.9	42.6	42.6	55.36[a]	18.84	47.86	19.00
	130% DSAA	42.7	42.5	42.5	46.82[b]	16.28	45.84	23.07
Pooled SEM		0.07	0.06	0.04	2.51	2.68	1.72	2.72
P Value								
Temperature		**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**
Diet		0.19	0.21	0.64	**0.02**	0.50	0.41	0.30
Temperature x Diet		0.15	0.65	0.64	0.91	0.75	0.37	0.86

[1] Values are means from five birds per pen or whole pen from 9 replicate pens from each interaction or 18 per main effect.

[2] TN = continuous 22 to 24°C; HS received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.

[a-b] Values in a column without common superscript letter are different ($P \leq 0.05$)

Table 5. The effect of SAA supplementation on hepatic GSSG, rGSH concentrations (measured 2 h after daily heat exposure on d 28 and 35) of broilers exposed to heat stress from 28 to 35 days of age[1]

Temperature program[2]	Diet[3]	28 days (Acute)			35 days (Chronic)		
		GSSG	rGSH	rGSH:GSSG	GSSG	rGSH	rGSH:GSSG
		——— nmol/g ———			——— nmol/g ———		
TN		418	3,779	9.07	378[b]	4138	11.81
HS		417	3,689	8.86	538[a]	4514	8.91
Pooled SEM		5.5	61	0.091	28	154	0.743
	Normal DSAA	417	3,722	8.93	482	4253	9.96
	130% DSAA	418	3,746	8.96	434	4399	10.75
Pooled SEM		5.5	61	0.091	28	154	0.743
P Value							
Temperature program		0.91	0.91	0.15	**≤ 0.01**	0.09	0.01
Diet		0.86	0.86	0.83	0.23	0.51	0.46
Temperature x Diet		0.44	0.44	0.08	0.13	0.17	**0.05**

[1] Values are means from one bird per pen from 9 replicate pens from each interaction or 18 per main effect.

[2] TN = continuous 22 to 24°C; HS received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.

[a-b] Values in a column without common superscript letter are different ($P \leq 0.05$)

Table 6. The effect of SAA supplementation on FITC-d in the serum (measured 2 h after daily heat exposure on d 28 and 35) of broilers exposed to heat stress from 28 to 35 days of age[1]

Temperature[2]	Diet[3]	FITC-d concentration		
		28 d	31 d	35 d
		ng/ml		
TN		113	110[b]	117
HS		142	116[a]	121
Pooled SEM		6	2	3
	Normal DSAA	135	113	119
	130% DSAA	119	112	118
Pooled SEM		6	2	3
P Value				
Temperature		≤ 0.01	**0.05**	0.34
Diet		0.05	0.67	0.81
Temperature x Diet		**0.05**	0.73	0.87

[1] Values are means from one bird per pen from 9 replicate pens from each interaction or 18 per main effect.

[2] TN = continuous 22 to 24°C; HS received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.

[a-b] Values in a column without common superscript letter are different (P ≤ 0.05)

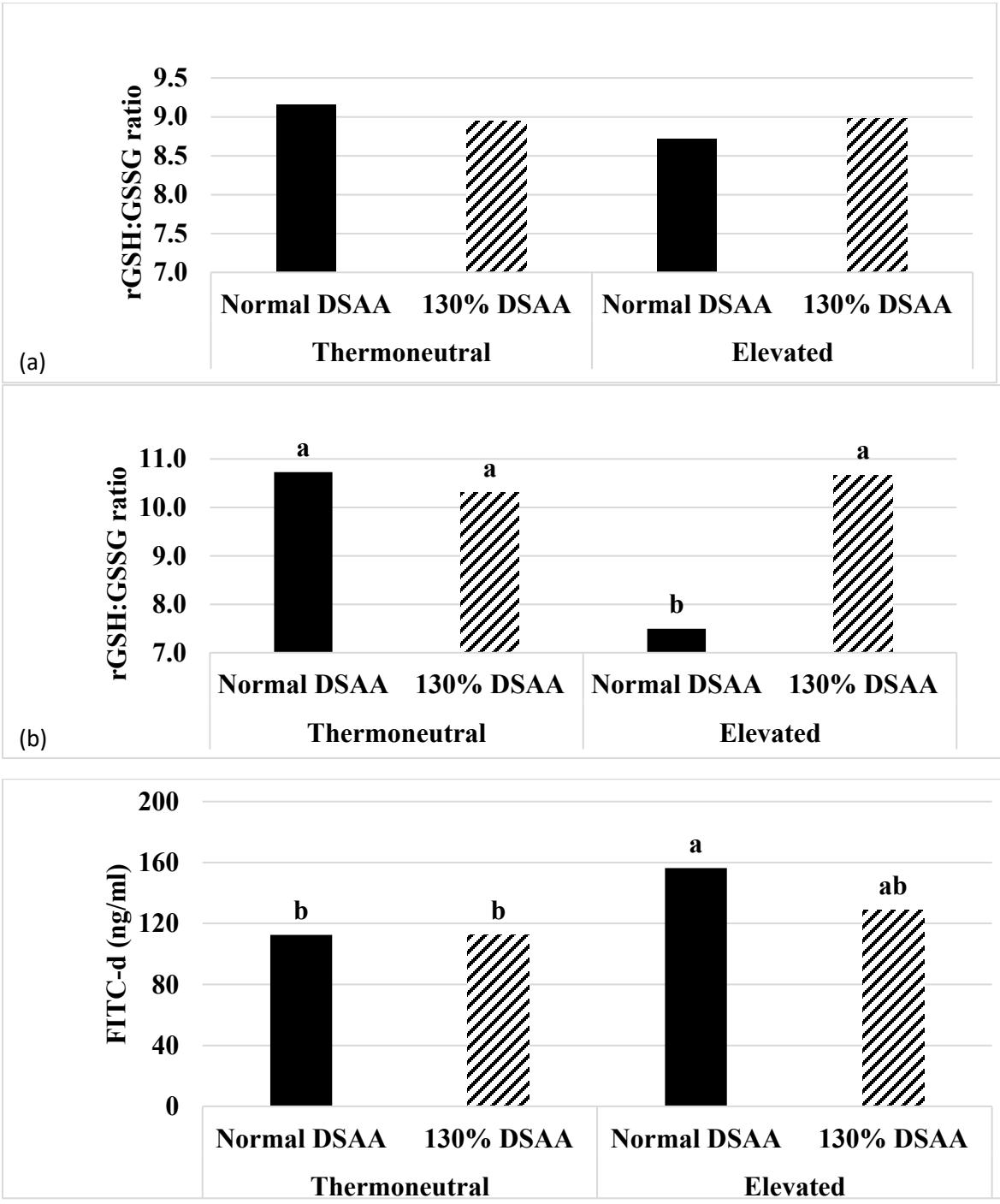

Figure 1. The Effect of a SAA supplementation on (a) reduced glutathione to oxidized glutathione ratio in the liver at 28 days of age sampled 2 hours after the initiation of heat stress (P = 0.08; SEM = 0.11), (b) reduced glutathione to oxidized glutathione ratio in the liver at 35 days of age sampled 2 hours after the initiation of heat stress (P = 0.05; SEM = 0.80), and (c) FITC-d concentration in the serum at 28 days of age 2 hours after the initiation of heat stress (P = 0.05; SEM = 8) of broiler chickens exposed to heat stress from 28 to 35 d

Scientific section: Management and Production

CHAPTER 6: EFFECTS OF A DIRECT FED MICROBIAL (DFM) ON BROILER CHICKENS EXPOSED TO ACUTE AND CHRONIC CYCLIC HEAT STRESS IN TWO CONSECUTIVE EXPERIMENTS ON REUSED LITTER

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ABSTRACT

Two consecutive 35 d experiments were conducted to investigate the effects of a multi-strain DFM fed continuously to broiler chickens exposed to heat stress from 28 to 35 d on cloacal temperature, broiler performance, body composition, ileal digestibility, and intestinal permeability. The treatments were arranged as a 2 x 2 factorial with temperature: Elevated (HS: 33 ± 2°C for 6 hours and 27.7°C for the remaining 18 hours from 28 to 35 days of age) and Thermoneutral (TN: 22 to 24°C over the entire 24 hour day from 28 to 35 days of age) and diet: corn-soybean meal based with and without DFM (3-strain *Bacillus; Enviva® PRO*) fed over the entire 35-d period as the two factors. Experimental diets were formulated to meet all nutrient recommendations based on breed standards using a starter (0 to 10 d), grower (10 to 21 d) and finisher (21 to 35 d) period. For each of the 2 experiments, 648 Ross 708 broiler chicks were allotted among the treatments with 9 replicate pens of 18 broilers. In both experiments, cloacal temperatures were increased ($P \leq 0.05$) in the broilers subjected to the HS treatment at both 28 d (acute) and 35 d (chronic). Supplementing birds with DFM reduced cloacal temperatures in Experiment 1 at 28 d ($P \leq 0.05$), but not at the other time periods. The HS treatment reduced body weight gain and lean tissue accretion from 0 to 35 d in both experiments ($P \leq 0.05$). In Experiment 2, when the litter was reused BWG was increased by 36 g/bird with supplementation of DFM compared to no supplementation ($P \leq 0.05$). Ileal digestibility at 28 d (2 hr post HS) was improved with DFM supplementation in both experiments ($P \leq 0.05$). Serum FITC-d increased with HS at both 28 and 35 d ($P \leq 0.05$). Serum FITC-d was generally decreased with DFM at 28 d ($P \leq 0.05$). Overall, the results suggest that HS reduced broiler performance and DFM treatment improved intestinal permeability and nutrient digestibility responses to HS in both experiments but did not improve performance until built up litter was used in Experiment 2.

Key words: Heat stress, broiler, DFM, intestinal permeability

INTRODUCTION

Elevated temperatures can disrupt thermoregulation and homeostasis of poultry potentially resulting in lost performance and livability. Although published nearly 20 years ago, the most recent estimates for economic losses from HS in the poultry industry suggest an annual 128-million-dollar loss (St. Pierre et al., 2003). Growing chickens exposed to HS will reduce feed intake representing lost growth potential and further shift those nutrient resources from growth to thermoregulation (Dale and Fuller, 1979). Therefore, understanding and finding mitigation strategies for the negative effects of HS has received extensive attention to reduce losses both from an economic and an animal welfare perspective.

As a response to elevated temperatures, chickens demonstrate behavioral responses such as increasing the surface area of exposed skin by spreading and fluffing their wings, digging into the litter to find a cooler surface, decreased activity and feed intake, and increased water intake (Mashaly et al., 2004; Quinteiro-Filho et al., 2010; Mack et al., 2013). As birds lack sweat glands, they use the panting mechanism with increased respiration rate which will result in increased water evaporation from the surface of the lungs reducing body temperature (Teeter et al., 1985). The increased respiration rate will result in excessive CO_2 losses which results in a decrease in the partial pressure of CO_2 in the blood and causes bicarbonate to disassociate and resulting in increased blood pH referred to as respiratory alkalosis (Koelkebeck and Odom, 1994; Barrett et al., 2019). The increased energy demand from active cooling methods (panting) during heat stress can also cause an increase in ROS resulting in DNA damage, lipid peroxidation, and protein oxidation (Quinteiro-Filho et al., 2010). Heat stress can disrupt tight junction proteins in the intestine which could result in increased intestinal permeability of pathogens and metabolites into circulation causing further loss of performance or even disease (Quinteiro-Filho et al., 2010; Wu et al., 2018). Heat stress has also been associated with changes in the microflora of the

intestine including a reduction in *Lactobacillus* and *Bifidobacterium* and an increase in coliforms and *Clostridium* (Song et al., 2014). These responses to elevated environmental temperature can result in decreased live performance, poor feed efficiency, and increased mortality in broilers (Deeb et al., 2002; Imik et al., 2012; Sohail et al., 2012).

One of the possible strategies to ameliorate the negative effect of HS is supplementing DFMs to poultry diets. These live and beneficial microbes have become a more common feed supplement in the poultry industry as an alternative to growth promoting antibiotics in antibiotic free production systems. The supplementation of DFMs can modulate the microflora within the host resulting in an improved intestinal balance and health of the bird (Fuller, 1989). Continuous feeding of DFMs results in an altered litter microbial community (Pedroso et al., 2013; Li et al., 2014). *Bacillus* spp., a commonly fed DFM, can increase the production of digestive enzymes which can enhance nutrient digestibility as well as inhibit pathogenic bacteria such *Clostridium perfringens* and *E. coli* (Wang et al., 2015; Whelan et al., 2019). Additionally, DFMs can improve intestinal morphological measurements such as villus height and villus height to crypt depth ratio indicating an increased available surface area for absorption of nutrients (Forte et al., 2016; Song et al., 2019). In laying hens, *Bacillus licheniformis* supplementation reduced serum corticosterone compared to control diets without supplementation which indicates a reduced stress response after 6 days of HS (Deng et al., 2012). This reduction in corticosterone was linked to a reduction in serum interleukin-1 and tumor necrosis factor α levels with supplementation of the DFM during HS. These cytokines have been linked to a reduction in feed intake and increased resting energy expenditure, gluconeogenesis, and glucose oxidation (Klasing, 1988). Consequently, the inclusion of a DFM during HS might alleviate the stress response during HS by reduction in cytokine production and exclusion of intestinal pathogens

improving live performance and livability. The objectives of the current experiments were to determine the effects of a DFM on broilers exposed to a cyclic HS over two consecutive flocks on broiler performance, cloacal temperature, body composition, ileal energy digestibility, litter bacterial counts and intestinal permeability as indicated by serum FITC-d.

MATERIALS AND METHODS

BROILER MANAGEMENT

All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech (Blacksburg, VA). Two consecutive 35 d experiments were conducted to investigate the effects of a multi-strain DFM (3-strain *Bacillus; Enviva® PRO*) on broiler chickens exposed to HS from 28 to 35 d. The treatments were arranged as a 2 x 2 factorial with temperature: HS (33 ± 2°C for 6 hours and 27.7°C for the remaining 18 hours from 28 to 35 days of age) and TN (between 22 to 24°C for 28 to 35 days of age) and supplementation with DFM over the entire 35-d growing period as the two experimental factors. For both experiments, 648 Ross 708 broiler chicks were allotted to the 4 treatments with 9 replicate pens of 18 broilers in two rooms. New pine shaving were used in the first experiment as bedding material. Stocking density was maintained at 548 cm^2/bird from 0 to 28 days of age and 823 cm^2/bird from 28 to 35 days of age in the 92 x 107-cm pens. Broilers were provided *ad libitum* access to experimental feed and water. Until HS treatments were initiated, temperatures were maintained according to breeder specifications based on the age of the birds which ranged from 30°C at placement to 22°C at 28 days of age (Aviagen, 2018). From 28 to 35 days of age, the above noted temperature treatments were applied using constant flow of room temperature air and a heater (TPI Corporation model# F3F551QT, 114 Roscoe Fitz Rd, Gray, TN 37615). Ventilation was

maintained using mixer fans to minimize difference in temperature across the length of the room. Continuous lighting was provided from 0 to 3 d of age, then the lighting was adjusted to provide 20 hours of light and 4 hours of darkness from 3 to 35 days of age according to the commercial management guide (Ross 708 management guide). Health checks occurred twice daily and when mortality was discovered, they were removed from the pen, weighed, and recorded. Broilers were culled during HS when they showed signs of heat distress. A sampling was conducted during what is referred to as the acute phase at 28 days of age 2 hours after the initiation of HS. The chronic phase sampling was conducted at 35 days of age 2 hours after the initiation of HS. The same litter was reused within the same pen in the second experiment. The litter was mixed with a shovel and allowed to dry within the same pen to maintain consistency due to possible differences in the litter due to DFM treatment. There was a 7-d downtime period between experiments.

DIET FORMULATION

A single experimental starter (0 to 11 d), grower (11 to 21 d), and finisher (21 to 35 d) diet was formulated to meet or exceed the nutrient requirements according to breeder recommendations (Table 1: Aviagen, 2018). A basal diet was generated and then equally split and 500 g/ton of a multi-strain DFM (3-strain *Bacillus; Enviva PRO*) was added on top of one of the diets and the other left alone to generate the two experimental diets for each phase. Finisher diets were formulated with 0.30% titanium dioxide as an indigestible marker. Starter diets were provided in crumble form and grower and finisher diets in pellets.

CLOACAL TEMPERATURE AND BLOOD CHEMISTRY

Cloacal temperature was measured at 28 and 35 days of age in each experiment from 5 broilers per pen. Cloacal temperature measurement was accomplished by inserting a thermometer (DeltaTrak MDL11064) 2 cm into the cloaca of each broiler. One ml of blood was collected from the brachial vein from one broiler per pen approximately 2-3 hours after the initiation of HS. Blood collection was accomplished using a 3 ml syringe with a 23-gauge x 1.6 cm needle with EDTA as an anticoagulant. The collected blood was immediately analyzed using an Abaxis iSTAT 1 handheld analyzer (Abbott healthcare, Princeton, New jersey) with a CG8+ cartridge (Abaxis item number 600-9001). Analyzed parameters included ionized calcium (iCa), pH, partial pressure of carbon dioxide (PCO_2), partial pressure of oxygen (PO_2), Total carbon dioxide (TCO_2), and bicarbonate (HCO_3).

BROILER PERFORMANCE AND BODY COMPOSITION

Individual body weights and feed offered and refused per pen were measured at hatch, and 11, 21, 28, and 35 d of age including all feed changes and the initiation of HS. Body weight gain and feed intake were calculated by difference of final weight and initial weight. Body weight gain and feed intake by pen along with mortality weight gain was used to calculate mortality corrected feed conversion ratio (FCRm), which is expressed as a ratio of feed consumed to weight gained by adding the pen mortality body weight gain to pen bird body weight gain. Body weights, feed intake and FCRm were calculated and analyzed over the 0 to 28, 28 to 35 and 0 to 35 d periods. After placement of chicks on experimental treatments on d of hatch, 30 remaining chicks were euthanized and frozen for later analysis. On the morning of d 36, 5 broilers per pen were euthanized, defeathered and frozen for later analysis. Both the day old and 36-day old sampled birds were allowed to come to room temperature for Dual-energy X-

ray Absorptiometry (DXA) with a Lunar Prodigy machine (GE Lunar, GE Healthcare, Waukesha, WI). These scans were utilized to measure fat and lean content of carcasses. The percentage of carcass fat and lean were used with body weight to calculate body fat and lean tissue at hatch and 36 days of age. The differences between the two sampling days were divided by the number of days to calculate a daily fat and lean accretion value between hatch and 36 d (Tran et al., 2021).

ILEAL ENERGY DIGESTIBILITY

Five broilers from each replicate pen were randomly selected and euthanized on d 28 and 35 for ileal digesta collection. Contents from the posterior half of the ileum as defined by Meckel's diverticulum to the ileal-cecal junction were collected by flushing the contents into a bag using distilled water. The ileal contents were pooled by pen and collected into WHIRL-PAK bags and frozen until analysis. Ileal samples were dried in a forced air oven at 65 °C for 24 hours. Dried ileal content and feed were ground using a cyclone mill with a 2-mm screen. Gross energy was measured in duplicate using a Parr 6400 bomb calorimeter. Feed and ileal samples were digested in sulfuric acid to determine amount of titanium in the sample using the methods of Short et al. (2015). Samples were analyzed by inductively coupled plasma atomic emission spectrometer at 336 nm to determine the concentration of titanium.

INTESTINAL PERMEABILITY

Serum concentrations of FITC-d were used to estimate intestinal permeability 2 h after HS exposure on d 28 and 35 using the methods of Baxter et al. (2017). Briefly, one broiler was randomly selected per replicate pen and was orally gavaged with FITC-d (8.32 mg/kg body weight) dissolved in double distilled water 1 h before the same chick was sampled for blood

collection from the brachial vein. These blood samples were stored in serum tubes and allowed to clot for 4 h at room temperature in dark conditions. Serum was isolated from the blood by centrifuging tubes at 2000 × g for 10 min at 4°C. Serum was then removed and diluted 1:5 in sterile 0.9% saline to a total volume of 100 µl in 96 well flat bottom black plate. FITC-d was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using multi-mode plate reader (Infinite M200 Pro, Tecan, Morrisville, NC). Serum fluorescent concentrations were then determined using a standard curve and sera of chickens not given FITC-d but directly spiked in the sera.

STATISTICAL ANALYSIS

Data were analyzed as a 2 x 2 factorial with the main effects being the HS and DFM within each experiment in JMP 14. The normal distribution of the data was verified by assessing the normal quantile plots in JMP 14. Student's t-test was used to separate the significant least squares means with the probability set at $P \leq 0.05$. Treatments were randomly blocked within rows that were used as the random variable in the analyses.

RESULTS AND DISCUSSION

CLOACAL TEMPERATURE AND BLOOD CHEMISTRY

In order to confirm the HS, cloacal temperatures were taken at the acute and chronic stages of heat stress 2 hours after heat stress was initiated (Table 2). No interactions were observed on cloacal temperature in either experiment or time point during HS ($P > 0.05$). HS resulted in elevated cloacal temperatures both at 28 and 35 days of age ($P \leq 0.01$) confirming the exposure of birds to HS. In the first experiment, there was a reduced cloacal temperature at 28 days during the acute response with the supplementation of the DFM ($P \leq 0.05$). No differences

were noted at 35 days in the first experiment or at either 28 or 35 days in the second experiment with the DFM treatment ($P > 0.05$). Previously, Ross 708 broiler subjected to a cyclic HS at 32°C for 10 hours per day demonstrated a reduction in panting and wing spreading with the supplementation of a *Bacillus subtillis* based DFM (Wang et al., 2018). Reduction in panting and wing spreading could indicate a lower stress response in DFM-fed broilers compared to non-DFM fed broilers. The inconsistent response in these experiments suggest that DFM treatment can possibly modify HS responses, but several factors can influence the response as well including strain of microbes utilized, environmental conditions including intensity and length of heat exposure, relative humidity, size, and age of the broilers.

No significant interactions were observed for blood chemistry at 28 days (acute bird response to HS) in Experiment 1 (Table 3). Heat stress resulted in a decrease in pCO_2 compared to TN ($P \leq 0.01$) which resulted in an increase of blood pH ($P \leq 0.01$). Furthermore, there was an observed decrease in ionized calcium (iCa) with HS compared to TN treated broilers ($P \leq 0.01$). Previous research has indicated that elevated blood pH results in the binding of iCa to proteins such as calbindin reducing availability of Ca to the bird (Etches et al., 2008). At 35 days, blood pCO_2 was still reduced ($P \leq 0.01$), and blood pH tended to be elevated ($P = 0.06$). Furthermore, there were two interactions observed for HCO_3 and total blood carbon dioxide (TCO_2) at 35 d of age. Bicarbonate and TCO_2 were both reduced with HS compared to TN without DFM supplementation and the supplementation of the DFM was able to partially ameliorate those effects which is important for reducing the effects of respiratory alkalosis within the body during HS (Figure 1; $P \leq 0.05$). A prebiotic and probiotic mixture supplemented to Ross 708 broilers subjected to a cyclic HS at 32°C for 10 hours per day from 15 to 42 d of age reduced behaviors associated with heat loss including panting and wing spreading (Mohammed et al., 2018). It was

speculated that this response was mediated through the microbiome resulting in improved regulation of the hypothalamic-pituitary-adrenal axis and reduced stress responses (Mayer et al., 2015). An early experiment conducted with Arbor Acre chicks subjected to a continuous HS at 32°C for 3 weeks reported a decrease in PCO_2 and HCO_3 which resulted in a higher pH in the blood (Teeter et al., 1985). An experiment conducted previously with a leghorn line exposed to a continuous HS at 38°C for 4 hours and then maintained at 35°C from 14 to 41 days of age reported a reduction in PCO_2 which resulted in a decrease in pH but no difference was observed on HCO_3 or iCa (Wang et al., 2018). Another study where broilers were subjected to an acute HS at 35°C for 8 hours at 31 days of age reported a reduction in PCO_2 and HCO_3 which resulted in an increase in the pH of the blood but no reported effect on iCa (Beckford et al., 2020). These results were consistent with our findings for PCO_2 and pH but were not consistent with HCO_3 responses. Previous reports have suggested that this may be due to the fact that birds are efficient at reabsorbing bicarbonate from the blood (Toyomizu et al., 2005).

In Experiment 2, when broilers were raised on previously used litter, no interactions were observed for blood chemistry at either 28 or 35 d of age (Table 4; $P > 0.05$). Mostly consistent with Experiment 1, the acute response to HS reduced blood pCO_2, TCO_2, and HCO_3 ($P \leq 0.01$) resulting in an increase in blood pH ($P \leq 0.05$). At 35 d of age after a 7-d chronic exposure to HS, birds continued to show a decreased blood pCO_2, TCO_2, and HCO_3 ($P \leq 0.01$), but pH was better regulated ($P > 0.05$). No significant effects of DFM supplementation were observed on blood gas chemistry either in the acute phase at 28 d or after chronic HS exposure at 35 d of age ($P > 0.05$). Comparisons between acute and chronic HS effects in broilers have been limited, but in laying hens, birds adapt to the HS and the pH and pO_2 do go back to normal levels after 4 weeks of HS (Barrett et al., 2019); however, the PCO_2, HCO_3 and TCO_2 were still reduced as the

hen were still using panting as a major cooling mechanism. There are several potential explanations for the differences in laying hen and broiler responses to chronic HS including differences in adaptation time, age at exposure, growth rate, feed intake and body size.

BROILER PERFORMANCE AND BODY COMPOSITION

There were no significant interactions observed among DFM and HS for BWG or FCRm in either experiment (Table 5; $P > 0.05$). As expected, HS resulted in a reduction in BWG from 28 to 35 days of age in both experiments (130 and 64 g/bird, respectively; $P \leq 0.01$). Mortality corrected feed conversion ratio was worsened in broilers that were subjected to elevated temperature from 28 to 35 days of age in Experiment 2 compared to TN broilers ($P \leq 0.01$). Previous research has shown reduction in BWG and/or worsening of FCR when broilers were subjected to heat stress. One experiment conducted with male broilers that were subjected to cyclic HS from 35 to 41 days of age at either 31 or 36°C for 10 hours per day then reduced to the thermoneutral comfort level for the remaining 14 hours. They observed a reduction in BWG with both the HS temperatures; however, the FCR was only worsened when the HS temperature was maintained at 36 °C compared to the control (Quinteiro-Filho et al., 2010). Additionally, a 31°C HS temperature was found to reduce BWG from 35 to 41 d of age without differences in FCR compared to the control group (Quinteiro-Filho et al., 2012). Another experiment conducted with Ross 708 mixed sex chicks that were subjected to continuous HS at 32 °C from 0 to 42 days of age observed a reduction in BWG and an increase in FCR between 0 and 42 days of age but only observed the reduction in BWG from 0 to 21 days of age (Sohail et al., 2012). Taken together, the current and previous results suggest that BWG is more sensitive to HS than FCR. This could be due to an early response to HS being a reduction in feed intake to reduce metabolic heat

production, but not altering metabolism (Lagana et al., 2007). Once HS is more severe, metabolic mechanisms begin to break down and FCR is worsened (Zhang et al., 2020).

In Experiment 1, when all pens started with clean pine shavings there were no differences in body weight or FCRm over the 0 to 28 d period, prior to the increase in temperature ($P > 0.05$). This lack of response to DFM treatment in a clean or new litter environment has been reported elsewhere (Gunal et al., 2006; Shargh et al., 2012, Olnood et al., 2015). Conversely, in the second experiment FCRm over the 0 to 28 d period was worsened in the pens that were exposed to HS in the first experiment ($P \leq 0.05$). This might suggest that the HS treatments changed the quality of the litter or the litter microbiome changing the microbial balance in the body resulting in the reduced efficiency. In Experiment 2 with the reused litter, DFM treated birds resulted in an improved FCRm over the 0 to 28 d period. This suggests that the DFM might be more effective in a more challenging environment (Gil De Los Santos et al., 2005; De Oliveira et al., 2019; Whelan et al., 2019). An alternative hypothesis is that the DFM is improving the microbiome of broilers altering the litter or litter microbiome resulting in the improved performance. The improvements in BWG and FCRm over the 0 to 28 d period were not carried into the HS period from 28 to 35 days of age resulting in FCRm being the only improvement over the entire 0 to 35 d period ($P \leq 0.01$). Male Hubbard broilers raised in battery cages and subjected to a cyclic HS (35°C for 5 hours and 21°C for 19 hours) from 21 to 35 days of age resulted in an improvement in BWG with the supplementation of a *B. Subtilis* DFM during HS (Al Fataftah and Abdelqader, 2014). Another experiment utilizing male Cobb broilers raised in battery cages and subjected to a continuous HS at 35°C from 15 to 35 days of age supplemented with a *Lactobacillus* strain probiotic found improved BWG and FCR (Jahromi et al., 2015). Ross 708 broilers subjected to a cyclic HS at 35°C for 10 hours and 21°C for the next 14 hours from

15 to 43 days of age reported an improvement in BWG and FCR with the supplementation of a *B. Subtilis* DFM to broilers raised in floor pens and exposed to HS (Wang et al., 2018). Hubbard broilers subjected to a cyclic HS at 35°C for 8 hours and supplemented with a DFM consisting of several *Lactobacillus* strains did not find any effect on BWG but found an improved FCR with supplementation of DFM to broilers in floor pens (Ashraf et al., 2013). Conversely, another experiment utilizing Ross 708 broiler chickens subjected to a cyclic HS for 8 hours daily at 35°C from 22 to 42 days of age fed diets containing a *Lactobacillus* strain found no improvement in BWG or feed efficiency (Sohail et al., 2012). A follow-up experiment utilizing Hubbard broiler chickens subjected to a cyclic HS for 8 hours daily at 35°C from 22 to 42 days of age fed diets containing the same *Lactobacillus* strain found no improvement in BWG or feed efficiency (Sohail et al., 2013). A recent experiment utilizing Ross 308 broilers subjected to a 10-hour cyclic HS at 33°C reported no effect on BWG or FCR when supplementing diets with *Bacillus subtilis* and *lichenformis* (Song et al., 2014). The variation in responses to the DFM can be caused by duration of the HS and different modes of action among various DFM. Kazemi and coworkers (2019) reported that different strains of DFMs have different modes of actions with some acting to improve live performance while others could improve the intestinal microflora and histomorphology for better nutrient absorption.

There were no significant interactions observed among DFM and HS for lean or fatty tissue accretion in either experiment (Table 6; $P > 0.05$). A reduction in lean and fatty tissue accretion was observed in Experiment 1 when the broilers were subjected to elevated temperature compared to birds held at TN temperatures ($P \leq 0.05$). However, only lean tissue accretion was reduced in Experiment 2 ($P \leq 0.05$). with no effect observed on fatty tissue accretion ($P > 0.05$). An older report utilizing Arbor Acres males exposed to cyclic 35°C HS for

4 to 6 hours resulted in reduced breast weight at 49 d of age but no difference in abdominal fat (Smith, 1993). Similarly, Arbor Acre males that were subjected to a constant HS of 34°C from 35 to 56 days of age reported a reduction in breast meat yield as well as subcutaneous, abdominal, and intermuscular fat compared to a control group that was maintained at 21°C (Lu et al., 2007). The inconsistent response in the current experiment and in the literature suggest that energy deposition may be more complex than lean tissue accretion with additional factors playing a role in the responses.

ILEAL ENERGY DIGESTIBILITY

Ileal samples were taken 2 to 3 h after the initiation of HS instead of fecal samples to capture the immediate effects of acute and chronic heat stress on energy digestibility in an attempt to better understand both digestibility and intestinal health responses. No interactions were observed in Experiment 1 (Table 6; $P > 0.05$), but in Experiment 2 at the 28-d acute period there was an interaction between HS and DFM (Figure 1). In Experiment 1, acute HS at 28 days resulted in effects for both the HS and DFM as HS decreased and DFM increased ileal energy digestibility independently ($P \leq 0.05$). At 35 days, there were no significant effects of HS or DFM ($P > 0.05$); although in Experiment 2, under the reused litter conditions, DFM tended to increase ileal energy digestibility ($P = 0.10$). Heat stress effects on digestibility have been mixed. Male Cobb broilers subjected to constant temperatures of 32°C from 28 to 46 increased AME compared to control birds (Keshavarz and Fuller, 1980). Male broilers subjected to a continuous 32°C HS from 45 to 56 days of age increased AME compared to TN (Geraert et al., 1992). Conversely, continuous 32°C HS of male Cobb 500 broilers resulted in no difference in AME at 31 days of age (Rosa et al., 2007). A more recent experiment used male Cobb broilers under cyclic and continuous HS found no differences in AMEn from 39 to 42 days of age compared to

controls (de Souza et al., 2016). The measurement of AME gives both the energy that is directly digested by the bird, but also includes fermentation by microbes and subsequent passive absorption of energy rich volatile fatty acid metabolic waste products of fermentation. Therefore, the comparison of AME to ileal energy digestibility may not be the best comparison as reduced feed intake could increase retention time and alter fermentation adding additional factors that can confound the ability of the broilers to directly digest and absorb energy or nutrients from the diet. In this case, dry matter or amino acid digestibility might be a better comparison to understand the effects of HS on ileal energy digestibly. There are several reports that show continuous HS reduces both dry matter and amino acid and protein ileal digestibly (Geraert et al., 1992; Zuprizal et al., 1993; de Souza et al., 2016). However, cyclical HS had no effect on digestibility (de Souza et al., 2016). Considering these comparisons, it does appear that HS compromises the ability of broilers to digest and absorb energy and nutrients directly from the diet.

Similar to the current results, DFMs have be reported to have the ability to improve AME in nutrient deficient diets (Weallands et al., 2017; Nusairat and Wang, 2020). Again, this AME comparison may be less valuable as it adds additional factors to consider. More directly, both dry matter and crude protein digestibility were increased in Cobb 500 broilers subjected to a cyclic HS at 36°C for 7 hours per day and supplemented with a *Lactobacillus* and *Saccharomyces* based DFM (Attia et al., 2017).

There are several demonstrated modes of actions that would support the ability of DFMs to increase nutrient absorption as demonstrated in the current studies. *Bacillus* based DFMs have been reported as a principal source of microbial enzymes including amylases, proteases, lipases, and phytases, potentially increasing enzyme activity to increase nutrient absorption (Lattorre et al., 2016). Furthermore, DFMs have can also improve intestinal morphological measurements

such as villus height and villus height to crypt depth ratio resulting in an increased available surface area for absorption of nutrients (Forte et al., 2016; Song et al., 2019).

INTESTINAL PERMEABILITY

Fluorescein isothiocyanate-dextran (FITC-d) has been used widely in the literature as an indicator of intestinal permeability. During HS, disease challenge, or feed restriction there is disruption to tight junction proteins that are located within the epithelial cells in the intestine which allows translocation of pathogens or metabolites into circulation (Maejima et al., 1984; Koh et al., 1996). Broilers dosed with FITC-d 2 h after initiation of HS at 28 d showed increased intestinal permeability ($P \leq 0.05$; Table 7). An interaction between HS and DFM was observed at 28 days in Experiment 1 (Figure 1; $P \leq 0.05$). Heat stress increased serum FITC-d concentration compared to the TN group without DFM but supplementing the DFM in the HS group reduced the FITC-d similar to the TN group regardless of DFM. At 35 d of age during the chronic phase, there were no effects of HS or DFM on intestinal permeability as indicated by FITC-d concentration in the serum ($P > 0.05$). In the second experiment during acute HS at 28 d, HS increased the intestinal permeability compared to TN group ($P \leq 0.05$), but the addition of DFM did not reduce intestinal permeability as indicated by FITC-d concentration in the serum ($P > 0.05$). At 35 d, FITC-d serum concentrations were still elevated compared to TN group, but in this case the addition of the DFM tended to reduce the serum FITC-d concentration ($P = 0.06$). Heat stress has been associated with higher FITC-d due to oxidative stress and inflammation within the chicken intestinal tract (Quinteiro-Filho et al., 2010). Cobb 500 broilers exposed to continuous HS at 32°C from 21 to 42 d of age showed increased serum FITC-d concentration on both 35 and 42 d (Ruff et al., 2020). Investigation of the effects of a 32°C 2 h HS at 29 d of age on four different breeds of broilers chickens found that three of the breeds with HS had higher

FITC-d serum concentrations compared to TN groups (Tabler et al., 2020). Although somewhat inconsistent, DFMs were able to reduce serum FITC-d concentrations in 2 of the 4 time periods explored in the current research. This is one of the first reports of DFM effect on intestinal permeability in broilers under HS, but DFM treatment can affect intestinal permeability in other intestinal challenge models. Treatment of broilers with *Bacillus* species have been shown to reduce intestinal permeability 10 days after a *Salmonella* challenge (Adhikari et al., 2019). Cobb 500 broilers infected with *Salmonella Typhimurium*, *Eimeria maxima*, and *Clostridium perfringens* were treated with a *Bacillus* DFM resulting in a reduction in serum FITC-d concentrations at 21 days of age compared to a challenged group without DFM (Hernandez-Patlan et al., 2019). DFMs can increase the expression of tight junction proteins such as JAM2 and occludin within the intestine which have been linked with reduced the intestinal permeability (Gadde et al., 2017). This would indicate that DFMs could have a protective effect on tight junction proteins which resulted in reduced intestinal leakage in the current experiment

The cyclic HS used in this experiment where broilers were not restored to TN temperatures after the HS period had a negative impact on performance resulting in reduced BWG, and protein and fat accretion in Experiment 1 and BWG, FCR, and protein accretion in Experiment 2. Cyclic HS resulted in a typical HS response including increased body temperature. The broiler's response to this increased temperature was to use panting to increase evaporative cooling altering blood chemistry and ultimately resulting in blood alkalosis during the acute phase of HS. The effect of the DFM was only apparent on growth performance responses when broilers were raised on the used litter which suggests that DFMs might have more of a beneficial response with more challenging environments. The DFM did not have any effect on performance during the HS; however, DFM did result in improved blood chemistry,

ileal energy digestibility, and reduced intestinal permeability when broilers were exposed to a cyclic HS.

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Table 1. Formulation and nutrient profile of experimental diets for starter (0 to 11 d), grower (11 to 21 d) and finisher (21 to 35 d) diets fed to Ross 708 broilers and exposed to elevated environmental temperatures from 28 to 35 d.[1]

Ingredient	Starter	Grower	Finisher
	----------(%)----------		
Corn	57.10	60.18	61.68
Soybean meal (48% CP)	33.00	27.00	21.00
Dried distillers grains w/ solubles	4.00	5.00	6.00
Poultry byproduct meal	2.00	4.00	6.00
Soy oil	0.10	0.49	1.84
Sodium chloride	0.15	0.20	0.15
Sodium bicarbonate	0.30	0.14	0.18
DL-Methionine	0.33	0.27	0.24
L-Lysine•HCL	0.19	0.14	0.16
L-Threonine	0.13	0.20	0.06
Limestone	1.04	1.01	0.98
Dicalcium phosphate	0.91	0.75	0.59
Phytase[2]	0.02	0.02	0.02
Choline chloride (60%)	0.10	0.10	0.10
Titanium dioxide[3]	---	---	0.30
Vitamin premix[4]	0.63	0.50	0.50
Nutrient Profile[5]			
Crude protein	22.50 (21.81)	21.50 (21.25)	20.44 (21.00)
ME, kcal/kg	2950	3060	3160
Calcium	0.90	0.90	0.90
Nonphytate phosphorus	0.45	0.45	0.45
Crude Fat	3.60 (3.40)	4.93 (4.16)	6.34 (4.68)
Fiber	2.80 (2.70)	2.76 (2.40)	2.53 (2.10)
Dig. Met	0.62	0.56	0.53
Dig. Cys	0.33	0.31	0.30
Dig. Met+Cys	0.95	0.87	0.83
Dig. Lys	1.28	1.15	1.06
Dig. His	0.52	0.50	0.47
Dig. Trp	0.22	0.20	0.19
Dig. Thr	0.86	0.77	0.71
Dig. Arg	1.29	1.23	1.14
Dig. Iso	0.83	0.79	0.73

[1]DFM was added on top of basal diet at 500 g/ton

[2]Axtra Phy (500 FTU/kg) was formulated to provide 0.10% of nPP and calcium

[3]Titanium dioxide was added as an inert marker for digestibility determination

[4] Provided per kg of diet: vitamin A, 1,320,000 IU; vitamin D3, 440,000 ICU; vitamin E, 2860 IU; menadione, 176 mg; biotin, 6.6 mg; vitamin B12, 1.9 mg; choline, 71.5 g; niacin, 6.6 mg; pantothenic acid, 1.8 g; selenium, 40 mg; riboflavin, 880 mg; Cu, 4.4 g; Fe, 45 g; I, 135 mg; Mn, 44 g; Zn, 44 g; Co, 4.4 g.

[5] Values within parenthesis are analyzed values for complete diets

Table 2. The effect of direct fed microbal (DFM) supplementation on cloacal temperatures (measured 2 h after daily heat exposure on d 28 and 35) of age of broilers exposed to acute HS at 28 days and chronic heat stress at 35 d of age in 2 consecutive experiments using built up litter.[1]

Temperature program[2]	Diet[3]	Experiment 1 with clean pine shavings		Experiment 2 with reused litter	
		28	35	28	35
		——————————————————————°C——————————————————————			
TN		41.7[b]	41.8[b]	41.6[b]	41.6[b]
HS		43.5[a]	42.7[a]	42.9[a]	43.2[a]
Pooled SEM		0.052	0.041	0.049	0.068
	Control	42.7[a]	42.3	42.2	42.5
	Control + DFM	42.4[b]	42.1	42.3	42.3
Pooled SEM		0.052	0.041	0.049	0.068
P-value					
Temperature program		**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**
Diet		**≤ 0.01**	0.27	0.45	0.08
Temperature * Diet		0.28	0.23	0.81	0.74

[1] Values are means from one bird per pen from 9 replicate pens from each interaction or 18 per main effect.
[2] TN: thermoneutral = continuous 22 to 24°C; HS: heat stress received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.
[3] DFM = direct fed microbial.
[a-b] Values in a column without common supperscript letter are different (P ≤ 0.05).

Table 3. Effects of DFM supplementation on the blood chemistry (measured 2 h after daily heat exposure on d 28 and 35) of broiler chickens raised on clean pine shavings and exposed to heat stress from 28 to 35 d, Experiment 1.[1,5]

Temperature[2]	Diet[3]	pH		pCO2		HCO3		TCO2		iCa[4]	
		28 d	35 d	28 d	35 d	28 d	35 d	28 d	35 d	28 d	35 d
				—— mmHg ——		———— mmol/L ————					
TN		7.28[b]	7.10	50.5[a]	67.9[a]	23.9	21.1	25.4	23.2	1.39[a]	-
HS		7.44[a]	7.14	35.2[b]	58.8[b]	23.5	20.2	24.5	21.9	1.29[b]	-
Pooled SEM		0.018	0.016	2.00	2.00	0.61	0.43	0.59	0.44	0.016	-
	Control	7.36	7.11	43.2	65.2	24.1	20.6	25.4	22.4	1.35	-
	C + DFM	7.35	7.14	42.5	61.6	23.4	20.7	24.6	22.7	1.34	-
Pooled SEM		0.018	0.016	2.00	2.00	0.61	0.43	0.59	0.44	0.016	-
P value											
Temperature program		**≤ 0.01**	0.06	**≤ 0.01**	**≤ 0.01**	0.64	0.15	0.30	0.05	**≤ 0.01**	**-**
Diet		0.78	0.33	0.33	0.14	0.44	0.80	0.38	0.66	0.63	-
Temperature * Diet		0.24	0.60	0.60	0.51	0.17	**0.05**	0.13	**0.03**	0.63	-

[1] Values are means from one bird per pen from 9 replicate pens from each interaction or 18 per main effect.

[2] TN: thermoneutral = continuous 22 to 24°C; HS: heat stress received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.

[3] DFM = direct fed microbial.

[4] Value for iCa were not within the detectable range at 35 days of age

[5] Ionized calcium (iCa), pH, partial pressure of carbon dioxide (PCO_2), partial pressure of oxygen (PO_2), Total carbon dioxide (TCO_2), and bicarbonate (HCO_3).

[a-b] Values in a column without a common superscript letter are different ($P \leq 0.05$).

Table 3. Effects of DFM supplementation on the blood chemistry (measured 2 h after daily heat exposure on d 28 and 35) of broiler chickens raised on clean pine shavings and exposed to heat stress from 28 to 35 d, Experiment 2.[1,4]

Temperature[2]	Diet[3]	pH		pCO2		HCO3		TCO2		iCa	
		28 d	35 d	28 d	35 d	28 d	35 d	28 d	35 d	28 d	35 d
				— mmHg —		——— mmol/L ———					
TN		7.42[b]	7.41	35.0[a]	38.5[a]	22.8[a]	23.6[a]	23.8[a]	24.7[a]	0.56	0.54
HS		7.51[a]	7.41	26.8[b]	32.6[b]	20.7[b]	20.4[b]	21.6[b]	21.4[b]	0.41	0.62
Pooled SEM		0.026	0.017	1.78	1.71	0.50	0.39	0.52	0.41	0.071	0.075
	Control	7.45	7.42	32.0	33.6	21.5	21.5	22.5	22.5	0.54	0.57
	C + DFM	7.49	7.39	30.0	37.5	21.9	22.5	22.8	23.6	0.41	0.60
Pooled SEM		0.026	0.017	1.78	1.71	0.50	0.39	0.52	0.41	0.071	0.075
P value											
Temperature program		**0.03**	0.85	**≤ 0.01**	**0.02**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	0.40	0.53
Diet		0.27	0.39	0.40	0.12	0.55	0.11	0.65	0.08	0.65	0.79
Temperature * Diet		0.08	0.27	0.09	0.12	0.75	0.25	0.88	0.22	0.46	0.11

[1] Values are means from one bird per pen from 9 replicate pens from each interaction or 18 per main effect.

[2] TN: thermoneutral = continuous 22 to 24°C; HS: heat stress received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.

[3] DFM = direct fed microbial.

[4] Ionized calcium (iCa), pH, partial pressure of carbon dioxide (PCO_2), partial pressure of oxygen (PO_2), Total carbon dioxide (TCO_2), and bicarbonate (HCO_3).

[a-b] Values in a column without a common superscript letter are different ($P \leq 0.05$).

Table 5. Effects of DFM supplementation on Body weight gain and mortality corrected feed conversion ratio (FCRm) on broilers over the 0 to 35 d period when exposed to heat stress from 28 to 35 d.[1]

Temperature [2]	Diet[3]	Experiment 1 with clean pine shavings						Experiment 2 with reused litter					
		Body Weight Gain			FCRm			Body Weight Gain			FCRm		
		0 to 28	28 to 35	0 to 35	0 to 28	28 to 35	0 to 35	0 to 28	28 to 35	0 to 35	0 to 28	28 to 35	0 to 35
		(g)			(g feed/g gain)			(g)			(g feed/g gain)		
TN		1,641	665[a]	2,308[a]	1.443	1.603	1.416	1,291	611[a]	1,901	1.473[b]	1.728[b]	1.547[b]
HS		1,605	535[b]	2,140[b]	1.470	1.630	1.450	1,263	547[b]	1,809	1.548[a]	1.970[a]	1.660[a]
Pooled SEM		17	31	44	0.016	0.060	0.022	13	16	20	0.014	0.038	0.012
	Control	1,620	587	2,207	1.467	1.636	1.446	1,257[b]	586	1,843	1.533[a]	1.861	1.628[a]
	C + DFM	1,626	612	2,238	1.445	1.596	1.436	1,296[a]	572	1,868	1.488[b]	1.832	1.579[b]
Pooled SEM		17	31	44	0.016	0.060	0.02	13	16	20	0.014	0.038	0.012
P Value													
Temperature program		0.15	**≤ 0.01**	**≤ 0.01**	0.28	0.74	0.28	0.13	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**
Diet		0.82	0.56	0.89	0.84	0.67	0.35	**0.04**	0.55	0.38	**0.03**	0.60	**≤ 0.01**
Temperature * Diet		0.60	0.65	0.63	0.52	0.39	0.72	0.93	0.62	0.65	0.06	0.88	0.29

[1] Values are means from from 9 replicate pens from each interaction or 18 per main effect.

[2] TN: thermoneutral = continuous 22 to 24°C; HS: heat stress received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.

[3] DFM = direct fed microbial.

[a-b] Values in a column without common superscript letter are different ($P \leq 0.05$).

Table 6. Effects of DFM supplementation on the overall lean and fat tissue accretion and ileal energy digestibility (measured 2 h after daily heat exposure on d 28 and 35 to measure acute and chronic responses, respectively) of broiler chickens exposed to heat stress from 28 to 35 d in both experiments.[1]

Temperature[2]	Diet[3]	Experiment 1 with clean pine shavings				Experiment 2 with reused litter			
		Lean tissue accretion	Fat tissue accretion	Ileal Energy Digestibility		Lean tissue accretion	Fat tissue accretion	Ileal Energy Digestibility	
		0-35 d	0-35 d	28 d	35 d	0-35 d	0-35 d	28 d	35 d
		(g/d)		(%)		(g/d)		(%)	
TN		48.5[a]	11.5[a]	77.8[a]	78.5	42.6[a]	8.7	76.8	76.9
HS		45.8[b]	10.2[b]	73.4[b]	77.6	40.4[b]	9.0	79.0	77.8
Pooled SEM		0.49	0.17	0.81	0.83	0.52	0.22	0.51	1.50
	Control	47.0	10.7	74.1[b]	77.5	41.8	8.7	76.5	75.5
	Control + DFM	47.2	11.0	77.1[a]	78.6	41.5	8.9	79.3	79.2
Pooled SEM		0.49	0.17	0.81	0.83	0.52	0.22	0.51	1.50
P-value									
Temperature		**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	0.37	**0.02**	0.37	≤ 0.01	0.68
Diet		0.72	0.35	**≤ 0.01**	0.31	0.73	0.48	≤ 0.01	0.10
Temperature * Diet		0.84	0.70	0.69	0.38	0.61	0.84	**≤ 0.01**	0.29

[1] Values are means from one bird per pen from 9 replicate pens from each interaction or 18 per main effect.

[2] TN: thermoneutral = continuous 22 to 24°C; HS: heat stress received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.

[3] DFM = direct fed microbial.

[a-b] Values in a column without a common superscript letter are different ($P \leq 0.05$).

Table 7. Effects of DFM supplementation on FITC-d (measured 2 h after daily heat exposure on d 28 and 35 to measure acute and chronic responses, respectively) of broiler chickens exposed to heat stress from 28 to 35 d in both experiments[1]

Temperature program[2]	Diet[3]	Experiment 1 with clean pine shavings		Experiment 2 with reused litter	
		28	35	28	35
		ng/ml			
TN		114	104	125[b]	119[b]
HS		167	118	138[a]	128[a]
Pooled SEM		17	5	4	2
	Control	160	112	135	129[a]
	Control + DFM	120	111	128	118[b]
Pooled SEM		17	5	4	2
P-value					
Temperature program		0.03	0.10	**0.03**	**≤ 0.01**
Diet		0.09	0.83	0.21	**≤ 0.01**
Temperature*Diet		**0.04**	0.81	0.22	0.06

[1] Values are means from one bird per pen from 9 replicate pens from each interaction or 18 per main effect.

[2] TN: thermoneutral = continuous 22 to 24°C; HS: heat stress received 33°C for 6 hours and 27.7°C for the remaining 18 hours daily.

[3] DFM = direct fed microbial.

[a-b] Values in a column without common letter are different ($P \leq 0.05$).

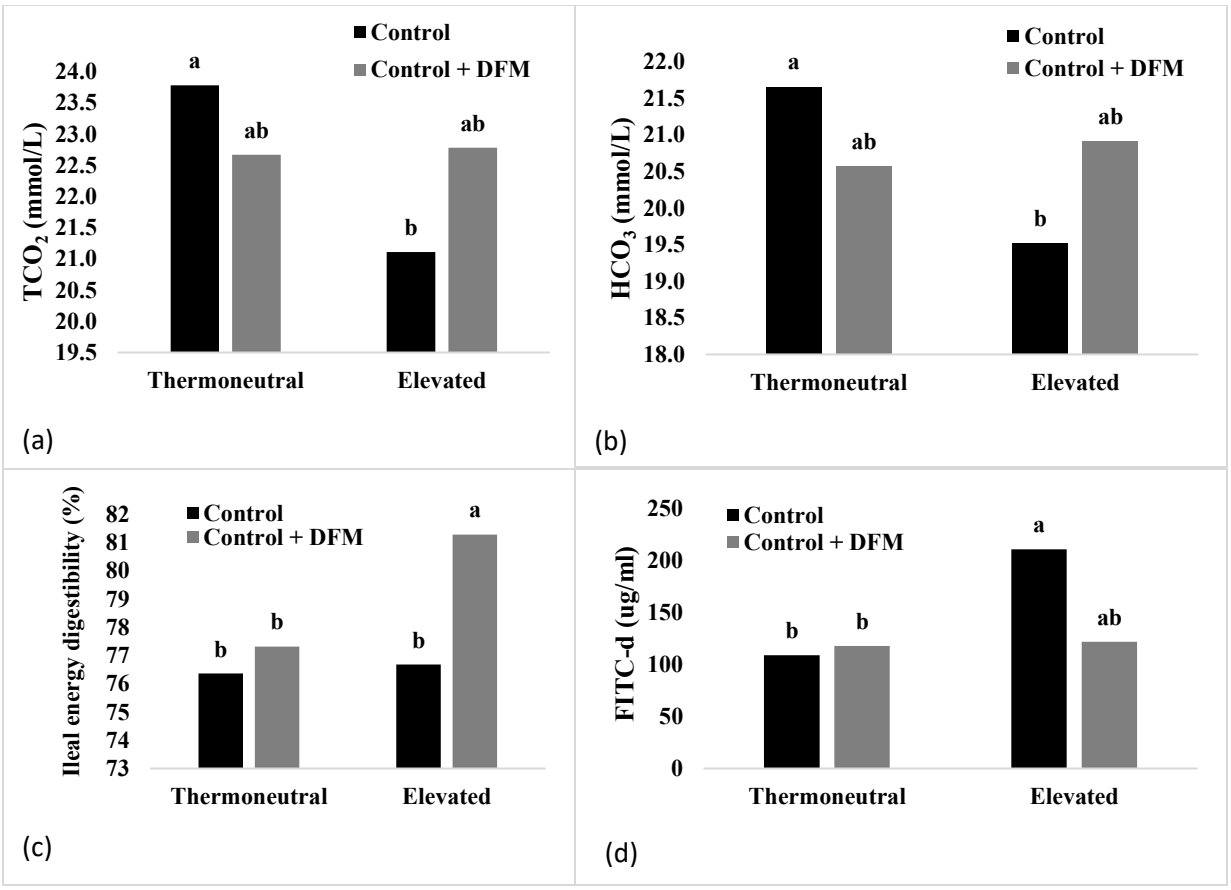

Figure 1. The Effect of Direct fed microbial supplementation on (a) Total carbon dioxide (TCO_2) in the blood in the first experiment at 35 days of age, (b) Bicarbonate (HCO_3) in the blood in the first experiment at 35 days of age, (c) Ileal energy digestibility in the second experiment at 28 days of age, and (d) FITC-d at 28 days of age in the first experiment of broiler chickens exposed to heat stress from 28 to 35 d in both experiments

CHAPTER 7: EPILOGUE

As body weight, egg production, and feed efficiency are improved through genetic selection, it is important that the amino acid requirements are updated to support the increase in productivity in laying hens and broilers. In laying hens, the requirement for Trp was previously met by adding additional soybean meal to the diet which might create an imbalance in other amino acids or cause more nitrogen to be excreted due to an overall increase in all amino acids. However, in the past few years a commercially available synthetic form of Trp (L-Trp) has emerged which allows poultry nutritionists to more closely formulate to the requirement for Trp. Therefore, the first objective of this dissertation was to determine the Trp requirement in first-cycle commercial laying hens in peak egg production. An experiment was conducted to evaluate the digestible Trp requirement of first cycle laying hens from 22 to 34 weeks of age. A total of 252 Hy-line W-36 laying hens were selected at 16 weeks of age and allocated by weight to seven dietary treatments supplemented with synthetic L-Trp to generate diets that provided 105, 119, 133, 147, 162, 176, and 190 mg digestible Trp on a daily basis over the experimental period Pullets were adapted to experimental diets by feeding complete diets that contained increasing amounts of corn gluten meal before the experimental period. The requirements for hen housed egg production (HHEP), egg mass (EM) and feed efficiency (FE) were estimated using three different models including linear and quadratic broken line and the quadratic polynomial model. The quadratic broken line model resulted in the best fit for all the parameters measured while the linear broken line had the lowest estimate for Trp requirement. Digestible Trp requirements were estimated to be 137, 183 and 192 mg/d for HHEP; 133, 180 and 183 mg/d for EM and 133, 177 and 173 mg/d for FE using linear broken-line, quadratic broken-line and quadratic polynomial analysis, respectively. Contrasting these results with previous requirement estimates for Trp in

the literature (Russell and Harms, 1999; Harms and Russell, 2000; Wen et al., 2019), we can conclude that the requirement for laying hens coming into egg production needs to be higher at peak production when laying hens are maximizing egg production and efficiency. The requirements estimated in this experiment provide a more recent estimate for Trp requirements during the peaking phase which allows nutritionists to formulate diets closer to the requirement for laying hens. The adaptation to experimental diets with increasing amounts of corn gluten meal allowed no reduction in feed intake when transitioning the hens to experimental diets. Therefore, when estimating amino acid requirements using unconventional ingredients to create a deficient diet, a 2-week adaptation period is recommended to allow adjustment to experimental diets. This allows more accurate estimates of the amino acid in question due to reduced drops in feed intake. The linear broken line is the classical method for modeling requirements; however, the quadratic broken line model resulted in a better fit. The quadratic broken line has advantages from the quadratic polynomial since it takes into account the concept of diminishing returns but is also similar to the linear broken line within the plateau region. The quadratic broken line model allows nutritionists to formulate based on maximum productivity of the flock. Future work should be focused on updating requirements for other limiting amino acids during this critical phase of production and the importance later in the production cycle. It is also important to investigate the requirement of Trp at different ages since the requirement will change depending on egg production. One of the limitations of this experiment is that it was set up as a requirement experiment, but poultry nutritionists formulate amino acids in diets based on a ratio to lys for simplicity.

Phase feeding could be used as an important tool in broiler production to reduce cost and improve growth and efficiency. Several studies have validated the concept of using regression

equations to predict short term requirements within each of the phases of production which would smooth the large drops in requirements seen in the transition between starter to grower to finisher (Emmert and Baker, 1997; Pope et al., 2002). The regression equations were developed based on the assumption that amino acid requirements decrease with age of broilers. Therefore, the second objective of this dissertation was to validate the assumption that Lys and SAA requirements decrease within the starter phase in a phase feeding scenario. Six short-term experiments were conducted to validate the hypothesis that Lys and SAA requirements decrease within the starter phase using 3-day periods from 2 to 11 d of age. In the first 3 experiments, 7 diets were generated by adding L-Lys to a lysine deficient basal diet in 0.10% increments, ranging from 0.85 to 1.45% Digestible Lys. In Experiments 4 to 6, 7 diets were generated by adding 0.07% increments of DL-methionine to a SAA deficient diet to produce diets ranging from 0.63 to 1.04% SAA. Requirements were estimated using the linear and quadratic broken line models. The Lys requirement was estimated to be 1.22, 1.17 and 1.16% for BWG and 1.31, 1.21, and 1.14% for FCR in Experiment 1, 2, and 3, respectively. The SAA requirement was estimated at 0.82, 0.81 and 0.94% for BWG and 0.82, 0.80, and 0.90% for FCR in Experiment 4, 5, and 6, respectively. The Lys requirement decreased with age from Experiment 1 to 3 as hypothesized. However, the SAA requirement decreased from Experiment 4 to 5 but increased from Experiment 5 to 6. This can be explained by the role of SAA in feather production from 7 to 11 days of age (Kalinowski et al. 2003; Zeng et al., 2015). As in the Trp experiment, the quadratic broken line model resulted in a better fit for the Lys and SAA requirements and therefore would be more accurate for determining requirements for maximum productivity. In these short term experiments, the breast weight results were not very applicable since the poultry industry does not process birds during the starter phase. An interesting approach would be to

continue the treatments up to processing age to determine the effect on breast weight. The battery caging system used in this experiment would not be appropriate since they cannot support broilers after 14 days of age and this experiment would have to be conducted in floor pens which would be more applicable to the industry. In this experiment, the length of the assay was shorter than 3-d when considering the sampling times so a longer assay time of 4-d would be appropriate to overcome the time lost to sampling and selection procedures. Further work would also need to be done to investigate the other limiting amino acids in broiler chickens using this experimental design to validate the hypothesis.

A third objective of this dissertation was to alter dietary SAA content of the diet of broiler chickens exposed to HS from 28 to 35 d to determine the effects on broiler performance, body temperature, intestinal permeability, and oxidative status. A total of 648 Ross 708 broiler chicks were allotted among treatments that were arranged as a 2 x 2 factorial with temperature: (Elevated and Thermoneutral) and 2 dietary concentrations of SAA (100 or 130% of breeder recommendations). The heat stress in this experiment was confirmed by increased cloacal temperature at 28, 31, and 35 days of age and panting at 32 and 33 days of age. As expected, HS resulted in reduced BWG and worsened FCR. In this experiment, the addition of SAA did not ameliorate these effects. Previous studies have reported that when diets are formulated to meet the SAA requirement for maximal growth there is no additional improvement in performance when fed in excess to broilers subjected to HS (Willemsen et al., 2011; Liu et al., 2019; Zeitz et al., 2020). Heat stress resulted in increased intestinal permeability at 28 and 31 days of age but not at 35 days of age which suggests that the intestine can adapt and respond to the environmental stimuli. More studies would need to investigate how this adaptation happens and what protective role heat shock protein have during HS. At 28 days of age, supplementation of

SAA reduced the permeability in the intestine within the HS group to be similar to the thermoneutral group. Additionally, SAA supplementation was able to restore the oxidative status of broilers subjected to HS at both 28 and 35 days of age. This may be due to the fact that SAA are precursors to an important antioxidant, glutathione, which can counteract the effects of ROS in the body which can damage DNA, lipids, and proteins. Increased reactive oxygen species can also result in increased intestinal permeability due to oxidative stress. Methionine can act as the primary donor for polyamine synthesis. Polyamines have been reported to be important in the stability of tight junction proteins within the intestine (Guo et al., 2005). Limited studies have been published on the effect of SAA on broilers exposed to HS. Investigating the effect of a continuous vs cyclic HS on SAA is important since previous research indicates that broilers do not acclimate similarly to a continuous vs cyclic HS. Previous experiments have used a supplementation of 20 to 30% of SAA requirements as their treatments within the HS period. Therefore, it is essential to determine what levels should be used to satisfy the requirement for performance and HS with least cost in mind. The effect of SAA on cyclic HS would also need to be determined over a longer experimental period and in broilers closer to an older market age to determine how long the responses on oxidative stress are maintained. The investigation of other strategies to ameliorate the effects of HS is crucial to improve performance and health of broilers in warm climates.

The final objective of this dissertation investigated the effects of a multi-strain DFM fed continuously to broiler chickens exposed to acute and chronic HS from 28 to 35 days of age in two consecutive experiments on built-up litter. Response criteria included broiler performance, blood chemistry, body composition, ileal energy digestibility, and intestinal permeability (FITC-d). In total, 648 Ross 708 broiler chicks were allotted among treatments that were arranged as a 2

x 2 factorial with temperature (Elevated and Thermoneutral) and diet (corn-soybean meal based with and without DFM) fed over the entire 35-d period as the two factors. The heat stress was confirmed by increased cloacal temperatures at both 28 and 35 days of age with an increase in the partial pressure of carbon dioxide and pH indicating respiratory alkalosis in the blood at 28 days of age. DFM supplementation reduced cloacal temperature at 28 days in the first experiment and restored the partial pressure of carbon dioxide and bicarbonate in the blood. This has been speculated to be related to reduced activity of the hypothalamic-pituitary-adrenal axis which can reduce the stress response (Mayer et al., 2015). Heat stress resulted in decreased BWG in the first and second experiment and increased FCR only in the second experiment. DFM supplementation resulted in improved BWG and FCR only in the second experiment when the litter was reused to increase the microbial load challenge. DFM supplementation also resulted in improved ileal digestibility in both experiments at 28 days of age. Serum FITC-d concentrations were increased with HS at 28 and 35 days of age in both experiments which indicated higher intestinal permeability. DFM supplementation decreased FITC-d concentrations in the serum when broilers were subjected to HS. Previous studies have reported that DFMs can have a protective effect on the intestinal epithelium and tight junction proteins resulting in decreased intestinal permeability (Gadde et al., 2017; Hernandez-Patlan et al., 2019). Future work should focus on understanding the dynamics of the communication between the intestine and the brain, which is a new field that could give us a better understanding of the influence of the microbiota on stress and disease. Comparing the previous literature to the current experiment, the effect of DFM supplementation on poultry subjected to continuous vs cyclic HS varies in responses and would need to be investigated together within the same experimental conditions and DFM strain. The cyclic HS temperature profile used in the current experiment was unconventional compared

to the literature as the temperature was not returned to thermoneutral conditions after the initial 6 hours of HS. This approach is applicable to practical HS conditions in the industry but not totally applicable for comparison with previous experiments using a cyclic HS. There was evidence to demonstrate an acclimation to HS for some of the parameters measured in the acute and chronic phases; however, further investigation is needed to determine whether the acclimation occurred and how long it takes for broilers to acclimate to cyclic HS conditions for all other parameters. In this experiment, the cyclic HS was maintained from 28 to 35 days of age which is applicable to small whole bird broiler production. In the United States, a large proportion of broiler production is targeting the large bird market (>4 kg) vs. the small bird market (<3 kg) to maximize breast meat yield, so it might be appropriate to look at the effects of a cyclic HS on broilers closer to this market age in which they may be more susceptible. An additional approach that could be used is subjecting broilers to a cyclic HS from 28 to 35 days of age then investigating responses at further market age to determine if broilers can compensate performance.

In conclusion, the linear broken line model resulted in lower estimates compared to the quadratic broken line in the first two chapters, but the quadratic broken line had a better fit to all the data. The highest requirement for Trp in mg/d was estimated for HHEP compared to egg mass and feed efficiency and the utilization of an adaptation period allowed for a more accurate requirement estimate. Digestible lysine requirements validated the phase feeding assumption that amino acid requirements decrease with the age of broilers during the starter phase when using regression equations to predict requirements over short periods of time: however, SAA requirements did not follow the same trend due to the role of SAA in other physiological functions. Supplementing direct fed microbials or sulfur amino acids to poultry diets can ameliorate the negative effects of heat stress in broiler chickens.

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