2013

Mitigation strategies to ameliorate acute and chronic heat stress utilizing supplemental methionine or embryonic thermal conditioning in chickens

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Mitigation strategies to ameliorate acute and chronic heat stress utilizing supplemental methionine or embryonic thermal conditioning in chickens

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Nutritional Sciences

Program of Study Committee:
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Iowa State University
Ames, Iowa
2013

DEDICATION

In loving memory of James Edward Ebidon and Arlene Alice Bolek.

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NOMENCLATURE

AIL	Advanced Intercross Line
BWG	Body weight gain
DLM	DL-methionine
ETC	Embryonic thermal conditioning
FCR	Feed conversion ratio
GSH	Glutathione; reduced glutathione
GPx	Glutathione peroxidase
GSSG	Oxidized glutathione
FI	Feed intake
HMTBA	2-hydroxy-4-methylthiobutanoic acid
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances
TGSH	Total glutathione

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my major professor, Dr. Michael E. Persia for all of his efforts throughout the past two years. Dr. Persia was one of my original inspirations for joining the poultry industry and later attending graduate school. I am sincerely grateful for having had the opportunity to work in his lab and be able to learn a great deal. Under his guidance and leadership, I pushed myself to accomplish more than I thought I ever could while becoming a better student in the process.

Thanks is also in order to my committee members, Dr. Nicholas K. Gabler and Dr. Susan J. Lamont. I truly appreciate their advice, patience, and willingness to help me with a myriad of questions regarding my research. Both Drs. Gabler and Lamont are truly masters of their craft and it a privilege to be associated with such tremendous scientific talent.

Many thanks to members of the Gabler, Lamont, and Persia labs. Upon my arrival to Iowa State, you all made me feel at home and welcomed me as a friend. It was a very rewarding experience to work alongside so many individuals with such tremendous potential. You give credence to the eminent reputation associated with the Department of Animal Science at Iowa State.

To my good friends Dr. Richard Faris, Dr. Elizabeth Bobeck, and Nestor Gutierraz, thank you all so much for all you have done for me. It would be next to impossible to find a more selfless group of individuals so willing to help others. Thank you for making my time here a fun one and giving me memories I will never forget.

A special thank you should be extended to Dr. Ganapathi Raj Murugesan. During my time as a master's student, Raj has been an invaluable resource, mentor, and friend. His guidance and unwavering support throughout my two years at Iowa State have made me a better student.

Lastly, I would like to thank my wonderful family for supporting through thick and thin: Mom, Dad, Jason, Kim, Isabella, and Christine. You have been my source of strength and continue to inspire me to be a better person. I love you all very much and count myself among the luckiest people on earth to have you in my corner.

ABSTRACT

Experiments were conducted exploring potential strategies to mitigate the negative effects of heat stress in chickens. In the first experiment, broilers were placed in environments which were heat treated (HT), thermoneutral (TN), or thermo-neutral coupled with pair-feeding (TN-PF). Birds were fed diets containing either DL-methionine (DLM) or 2-hydroxy-4-methylthiobutanoic acid (HMTBA) formulated to adequate or super-adequate levels of digestible sulfur amino acids (DSAA). Reduced (GSH), oxidized (GSSG), and total (TGSH) glutathione as well glutathione peroxidase (GPx) activity were measured to assess antioxidant capacity. Thiobarbituric acid reactive substances (TBARS) were measured to assess lipid peroxidation. Several blood variables were also measured. Responses were measured at 6 hours (acute) and 6 days (chronic). Growth over 6 days was impaired by HT ($P < 0.01$). Blood variables were altered during the acute phase. Birds fed DLM during the acute phase exhibited lower TBARS concentrations than those fed HMTBA ($P = 0.02$). Concentrations of GSSG were lower ($P < 0.01$) in birds receiving super-adequate DSAA than those receiving adequate DSAA in the acute phase. During the chronic phase, GSH and TGSH concentrations were higher ($P = 0.02$) in the HT group compared to the TN group. There were no effects on GPx activity. During the second experiment, broiler x Fayoumi crosses were incubated at a normal incubation temperature (NI; 37.8°C) or elevated incubation temperature (EI; 39.5°C) from embryonic day -12 to -4. Birds were then placed in TN or cyclical HT from 22 to 28 days of age. Growth performance and feed dry matter digestibility were assessed from 21 to 28 days of age. An interaction occurred where birds in the EI group in HT environments had higher

digestibility than those birds in the NI group held at TN temperatures. The third experiment followed the same procedures from the second experiment, except all were incubated normally, then divided into groups in which birds had parents that were incubated normally (PN) or parents incubated at elevated temperatures (PE) from the previous experiment. Heat treatment during the third experiment increased BWG. In summary, neither methionine supplementation nor embryonic thermal conditioning improved growth performance during heat stress.

CHAPTER 1

GENERAL INTRODUCTION

Heat stress is a significant issue affecting broiler chicken production in the United States as well as other parts of the world. Heat stress is known to cause reduced body weight gain (BWG) and feed intake (FI), as well as an increased feed conversion ratio (FCR) in broiler chickens. Exploring mitigation strategies for broilers in heat stressed conditions may have the potential to maximize economic gains for producers and improve animal welfare. The thesis research presented here examined two potential heat mitigation strategies: (1) Utilizing supplemental methionine as a nutritional intervention during heat stress and (2) utilizing embryonic thermal conditioning (ETC) to augment broiler chicken development, rendering them more resistant to the effects of heat stress. When feeding corn-soy broiler diets, supplemental dietary methionine is essential for maintaining the requirements for both methionine and cysteine in growing poultry in economic and environmentally responsible feed formulation. While cysteine has multiple biological functions, it is an important and often the limiting component of glutathione (gamma-glutamyl-cysteinyl-glycine, GSH), a tripeptide functioning as part of the antioxidant defense system in birds and mammals. Heat stress in known to increase the production of free radicals and reactive oxygen species (ROS), compounds which, when overproduced, can have deleterious effects on the constituents of biological tissues. The over-production of these reactive species can be increased through mitochondrial electron leakage (Ando et al., 1997), as well as increased activity of xanthine oxidase (Hall et al., 2000), and mechanisms mediated through cellular hypoxia (Hall et al., 1999). Given the importance of

methionine and cysteine's role in contributing to GSH production, increased dietary supplementation of digestible sulfur amino acids (DSAA) may provide additional substrate for the production of GSH during a heat stress challenge. Embryonic thermal conditioning may also provide a means through which poultry can be adapted to heat stress. Previous research has shown that, in some cases, ETC can positively affect post-hatch chicken growth performance as well as improving heat tolerance during periods of heat stress. The thesis research presented here is a culmination of three experiments examining the effects of methionine supplementation and embryonic thermal conditioning on the heat stress response in chickens and to determine the appropriateness of these approaches as heat stress mitigation strategies. The two-part hypothesis guiding this thesis research was:

1.) Super-adequate (20% above requirement) dietary supplementation of DSAA supplementation would maintain broiler antioxidant status and growth performance during periods of acute and chronic heat stress.

2.) Embryonic thermal conditioning would produce birds that would be better adapt to heat stress conditions. Furthermore, these phenotypic changes may be carried over to subsequent generations.

The first objective of this research was to determine the effects of varying DSAA supplementation from two methionine sources on growth performance, antioxidant status, and blood chemistry variables of broilers experiencing acute (6 hours) and chronic (6 days) heat stress. The second objective of this research was to determine whether there were any significant effects of ETC on growth performance and individual feed dry matter digestibility in experimental chicken crosses and whether these effects could be observed in the subsequent progeny.

CHAPTER 2

REVIEW OF THE LITERATURE

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Impact of Heat Stress on Growth Performance

Environmental heat stress has significant implications for broiler chicken welfare and performance, directly affecting the profitability of producers. An economic study conducted by St. Pierre et al. (2003) estimated that, in the United States alone, impaired growth performance and increased mortality due to heat stress costs producers $51 million annually. Elevated environmental temperature is an abiotic stress associated with well-documented effects on growth performance. Generally, these effects can be characterized as a reduction in body weight gain (BWG), feed intake (FI), gain:feed ratio or feed efficiency (FE), and increased mortality (Reece et al., 1972; Cooper and Washburn, 1998; Geraert et al., 1996; Mujahid et al., 2009). There are variations in the estimates for the ideal temperature range for broiler performance, probably due to differences in age, sex, and strain examined in the literature. A review by Charles (2002) estimates that broiler performance is optimal in a temperature range of from approximately 18-22°C. Temperatures beyond this point that approach the upper critical temperature can affect performance. The upper critical temperature can be defined as the temperature above which the body temperature increases until it reaches a point at which the bird expires (Daghir, 2008). Estimates for upper critical temperature in poultry vary as well, which can be dependent on other variable such as relative humidity (Lin et al., 2006). Lin et al. (2005) demonstrated that 4-week-old broilers kept at temperatures of 30°C and 60% humidity

facilitated heat transmission form the body core, but impaired it at 35°C. Other authors estimate that the upper critical temperature ranges from 29-32°C (Esmay, 1982; Hahn, 1982). To an extent, broilers are able to adapt themselves to a heat stress challenge through various mechanisms that include increasing peripheral blood flow to dissipate excess heat, decrease feed intake to reduce heat increment, as well as increase panting to facilitate evaporative cooling (Daghir, 2008).

Both constant as well as a cyclical heat stress, in acute and chronic settings, may induce these effects. For instance, Cooper and Washburn (1998) found that housing broiler chickens at a constant 32°C from 28 to 49 days of age significantly reduced BWG and FI, while increasing the feed:gain ratio (feed conversion ratio, FCR). Another study conducted by Geraert et al. (1996) demonstrated that chronic exposure to temperatures of 32°C from periods of 14 to 28 days of age as well as 27 to 41 days of age impaired performance in broiler chickens. Other studies attempt to more closely mimic climatic conditions, perhaps in accordance with local weather patterns, by adopting a pattern of cyclical heat stress throughout the duration of the experiment. As with chronically elevated environmental temperatures, cyclical heat stress can exert a negative influence on broiler performance as well. For example, Stillborn et al. (1987) found that broiler chickens exposed to cycling temperatures, ranging from 24° C to 35°C displayed impaired FI and BWG. Another study conducted by Balnave and Oliva (1990) found that broilers subjected to cycling temperatures with a range 25°C 35°C from 21 and 41 days of age showed a significant reduction in FI and BWG accompanied by a significant increase in FCR. These experiments provide evidence that both cyclical and well as constant heat stress may exert

negative effects on the growth performance of broilers, reducing the body weight at market age and impairing the efficiency of tissue accretion in relation to feed consumption.

Feed consumption is an important factor with regard to heat stress. It has been well documented that, during periods of heat stress, feed intake in reduced in broilers, limiting the intake of nutrients and therefore limiting growth performance (Garaert et al. 1996). This is likely due to the birds attempting to reduce the heat load within the body by reducing feed intake as increased digestion with feed is associated with increased heat production in the body (Daghir, 2008). However, this reduction in feed intake is not the only contributing factor to reduced growth performance during heat stress. Garaert et al. (1996) illustrated this point by utilizing a pair-fed group of broiler chickens in order to separate and quantify the effects of heat stress alone on growth performance. The heat treated group in this experiment was subjected to increased environmental temperature (32°C) from 14 to 28 d of age and gained 5.5% less weight than the pair-fed group, while the group from 14 to 28 d of age gained 22% less weight than the pair-fed group.

This demonstrates that heat stress impairs growth performance beyond a reduction in feed intake, presumably through other physiological mechanisms contributing to lost performance. Among these include a decrease in protein accretion, alterations in the intestinal microbiome, increased production of free radical species, and suppression of the immune system (Hall et al., 1999; Burkholder et al., 2008; Quinteiro-Filho et al., 2010). Each of these phenomenon are associated with poor growth performance and may play a role in contributing to the negative effects of heat stress.

Heat stress can impair the function of the gastrointestinal tract, compromising the barrier function of the intestinal epithelium and increasing the likelihood of pathogen

translocation. Several studies have clearly demonstrated these effects, using rodents as models. Lambert et al. (2002) showed that anestitized rats with a core temperature of 41.5°C showed a significant increase in intestinal permeability as assessed by plasma concentrations of FITC-dextran compared to rats with a core temperature of 37.5°C. The same study also found that subjecting everted intestinal sacs from rats to temperatures ranging from 41.5°C to 42°C significantly increased FITC-dextran permeability compared to temperatures of 37°C within 45 minutes of treatment application. Hall et al. (2001) observed a significant increase in serum concentrations of lipopolysaccharide (LPS), a toxic bacterial component, in anestitized rats heated to core temperatures of 41.5°C. Dokladny et al. (2006) showed that Caco-2 cell monolayers maintained at 41°C over a period of 24 hours displayed reduced transpeithelial resistance as well as increased paracellular permeability. Other data show that heat stress conditions also can increase the chance of pathogen attachment in the gastrointestinal tract. An experiment conducted by Burkholder et al. (2008) utilized market-age broiler chickens subjected to temperatures of 30°C for a period of 24 hours and showed an increased attachment of *Salmonella enterditis* in the ileum and altered the gastrointestinal microbiome. Compounding the fact of increased pathogen attachment and bacterial translocation, poultry can also suffer from impaired immune function during bouts of heat stress.

Illustrating this point is an experiment conducted by Quinteiro-Filho et al. (2010) in which broiler chickens were exposed to temperatures of either 31°C or 36°C for 10 hours a day from 35 to 42 days of age. Subsequent measurements of macrophage activity revealed that, compared to broilers raised at a control temperature of 21°C, broilers exposed to higher temperatures displayed decreased macrophage activity as illustrated in a reduction

in the oxidative burst in response to a *Staphylococcus aureus* challenge. Mashaly et al. (2004) demonstrated that laying hens exposed heat stressed condition not only displayed reduced growth performance and egg quality characteristics, but also reduced total white blood cell counts and antibody production, indicative of impaired immune function.

The experiments outlined here illustrate various mechanisms through which heat stress can exert detrimental effects on the growth performance of broiler chickens. Each of these effects affect the bird in a different manner, but all compound to reduce overall bird performance.

Impact of Heat Stress on Blood Variables

During periods of increased environmental temperatures, broiler chickens undergo various physiological changes, including alterations to blood flow and blood components. During heat stress, blood flow is modulated in order to dissipate excess heat, with an increase in circulation to peripheral tissues such as the comb or wattles during elevated environmental temperatures (Whittow, 1986). Several other blood parameters may be altered in response to high temperatures, especially during the onset of heat stress, or acute heat stress. Changes take place in the blood during periods of heat stress that reflect changes in respiration, electrolyte balance, serum metabolites, and pH (Toyomizu et al., 2005; Borges et al., 2003; Sahin et al., 2003). Several studies have examined the effect of high environmental temperatures on blood variables in various poultry species, with many of the effects on blood parameters consistent and well characterized. For example, a study conducted by Toyomizu et al. (2005) demonstrated that exposing broiler chickens to

temperatures of 36°C or 38°C for 120 minutes increased respiration rate. In the same study, a second experiment was conducted which again held broilers either at 38°C for 120 minutes or at thermoneutral temperature of 24°C. Birds held at 38°C had significantly increased blood pH, decreased blood $pCO_{2,}$ and decreased bicarbonate levels compared to the thermoneutral group held at 24°C. Another study conducted by Sandercook et al. (2001) found that male broiler chickens subjected to heat stress at both 35 and 63 days of age displayed a lower pCO_2 as well as an increase in blood pH. Panting can lead to an increase of CO_2 expelled from the lungs and, in turn, decreasing pCO_2 in the blood (Wang et al., 1989). As a result, decreased circulating hydrogen ions lead to an increase in plasma pH which is followed by respiratory alkalosis (Richards, 1970). Respiratory alkalosis has been associated with depressed growth in broiler chickens as well as impaired eggshell quality in laying hens (Lin et al., 2006).

Other changes associated with increased environmental temperatures include changes in electrolyte concentrations. Potassium (K^+) is the main cation in intracellular fluids and is involved in the various physiological processes including nervous conduction, muscle contractions, osmotic balance, synthesis of tissue proteins, enzymatic reactions, and acid-base balance (Borges et al., 2007). During respiratory alkalosis, the reduction of circulating hydrogen (H^+) ions may lead to reduced competition between K^+ and H^+ for urinary excretion (Borges et al., 2007). Thus, this may serve as a mechanism by which K^+ excretion is increased in broiler chickens during periods of high environmental temperatures. Sodium (Na^+) and chloride (Cl^-) are also important electrolytes affected by heat stress. While plasma concentrations of Na^+ generally decrease during periods of heat stress, Cl^- concentrations have been shown to increase (Borges et al., 2007) during heat

stress, which may be a mechanism to increase renal acidification as increases in Cl^- reduce H^+ excretion and bicarbonate reabsorption. However, chronic heat stress during a one week period has also been shown to decrease Cl^- concentrations (Salvador et al., 1999) as well. These findings imply that heat stress can have a significant impact on various blood parameters and may exert an effect on broiler performance.

The relatively consistent changes to blood variables during a heat stress challenge illustrated by these findings demonstrate the plausibility of utilizing changes in blood variables as a marker of heat stress.

Impact of Heat Stress on Body Protein Accretion

The lean composition of broiler chickens is of crucial economic importance to producers. Various studies present evidence showing the reductions in BWG, gains in skeletal muscle, and reduced proliferation of muscle satellite cells during periods of high environmental temperature. For example, periods of chronic heat stress have been found to not only decrease the rate of protein synthesis, but also increase the rate of protein degradation (Lin et al., 2006). Temim et al. (2000) found that birds kept at a constant 32° C temperature showed a decrease in body weight and growth rate. Concurrently, the study found that there was significantly reduced RNA and protein weight in the *Pectoralis major* muscle while RNA content was significantly reduced in the in the *Sartorius* and *Gastrocnemius* muscles. It was also determined that translational efficiency was also impaired in breast muscle tissue of the heat stressed birds. Furthermore, the effect of reduced protein synthesis seen in this study could not be remedied by increasing the crude

protein content of the diet. Geraert et al. (2006) showed that broilers kept at 32° C have not only reduced growth rates compared to their pair-fed counterparts (housed at 22° C), but also significantly reduced body protein content. Another study conducted by Cheng et al. (1997) showed that increasing environmental temperatures to 32.2°C decreased total body protein as well as decreased protein utilization as calculated as the percentage of total body protein to 7 wk protein intake. Mendes et al. (1997) found that broiler chickens subjected to cyclical heat stress 21-42 days of age displayed a decrease breast meat and leg quarter yield as well as dressing percentage. These studies clearly illustrate the significant affect heat stress exerts on muscle protein synthesis, reducing muscle protein accretion and effecting not only RNA content, but translational efficiency as well.

While high environmental temperatures can significantly reduce muscle protein accretion, they can also affect the integrity of existing skeletal muscle. Sandercook et al. (2001) illustrated that broiler chickens subjected to environmental conditions of 32°C for a period of two hours showed an increase in plasma creatine kinase activity, an indicator of skeletal muscle damage. There was also an increased incidence of breast muscle hemorrhages in heat treated birds compare to those raised at 21°C. These experiments demonstrate the negative effects that high environmental temperatures can exert on body protein accretion and utilization as well as muscle integrity in broiler chickens.

Increased Production of Free Radicals during Heat Stress

Elevated environmental temperatures are associated with several negative effects with regard to broiler performance and health. One of these mechanisms through which

heat stress is thought to have deleterious effect on an organism is through the production of free radicals and reactive oxygen species (ROS) within the body (Bruskov et al. 2002). Free radicals and ROS are compounds generated naturally within an organism during normal biological processes and are essential for several of the body's processes, including immune function (Valko et al., 2006). However, pathological conditions or environmental stressors can shift this balance to a more prooxidative state and, in turn, have negative consequences for the organism (Valko et al., 2006). An increased production of these compounds can cause damage to the constituents of various biological tissues including lipids, protein, and DNA (Fang et al., 2002). High environmental temperatures may increase the production of these compounds through various mechanisms. Mujahid et al. (2005) demonstrated that high environmental temperature is associated with a direct increase in the production of superoxide in broiler skeletal muscle. This increase in ROS production may be due to the disruption of the assemblies of the electron transport chain during periods of increased temperature (Ando et. al., 1997). Although this is a well-known phenomenon in which reactive oxygen species are produced during heat stress, several other mechanisms are present as well. For example, damage caused to lipids by free radicals and ROS themselves can lead to the formation of lipid peroxyl radicals, which in turn may lead to further lipid oxidation through propagation reactions (Benzie et al., 1996). Other means through which heat can increase the production of these reactive compounds include those mediated through ischemia and hypoxia. Mishra et al. (2000) demonstrated that hypoxia in cerebral tissue is associated with an increase in the concentration of nitric oxide, which can react with superoxide to form peroxynitrite, a mechanism in which the author theorized to cause neuronal injury. With respect to

ischemia, heat stress has been linked to a reduction of blood flow to splanchnic viscera, which is associated with the production of various radical species (Hall et al., 1999). This includes the gastrointestinal tract, where free radical production can occur directly, a point of particular importance in the context of growth performance and nutrient utilization. A study conducted by Flanagan et al. (1998) demonstrated the direct production of free radicals in rat epithelial cells exposed to temperatures of 45° C for a period of 20 minutes utilizing electron paramagnetic resonance spin trapping. As with other tissues, the production of free radicals in the intestinal epithelium is also associated with cell damage and reduced intestinal integrity (Banan et al., 2000). A compromised intestinal epithelium is associated with poor broiler performance (Quinteiro-Filho, et al. 2010) and may allow for increased bacterial translocation across the intestinal epithelium.

Sources of Methionine and Patterns of Absorption

Methionine supplementation plays a crucial role in ensuring adequate nutritional status of broiler chickens. It is generally considered the first limiting amino acid in corn-soy based poultry diets and is necessary for various functions within the body, including muscle protein accretion and antioxidant production (Métayer et al., 2007). Demonstrating its importance, deficiencies in methionine, as well as cysteine, cause a more pronounced depression in body weight gain and protein accretion than even diets which are deficit in lysine or histidine in young chicks (Kino and Okumura, 1986). Decreased growth seen in chicks fed methionine and cysteine free diets is primarily caused by decreased rates of protein synthesis associated with lower mRNA efficiency, suggestive of translational regulation (Metayer et al., 2008). This is a logical association due to the fact that

methionine plays a critical role in the initiation of protein synthesis in eukaryotic organisms (Drabkin and Rajbhandary, 1998). Other studies have shown an increase in lean tissue accretion concurrently with increased methionine supplementation in broiler chickens (Jensen et al., 1989). As is such, methionine is generally supplemented to some extent within broiler diets to ensure an adequate level of sulfur amino acids within the diet. Two commonly used forms of this methionine include DL methionine (DLM) and 2-hydroxy-4-methylthiobutanoic acid (HMTBA), a hydroxy acid analog structurally similar to that of methionine. While DLM is generally supplemented in a powder form to be mixed into diets, HMTBA is generally manufactured in liquid form. Both molecules are absorbed through different routes and are metabolized in different fashions within the body.

Methionine can be absorbed directly by the bird mediated through various active transport system across the apical portion of the intestinal epithelium, consisting of both sodium dependent and sodium independent transporters (D'Mello, 2003). While the L-configuration must be used for protein synthesis, methionine can be fed as a racemic mixture, utilizing either D or L methionine as the D enantiomer can be converted to the L form and utilized directly by the birds (D'Mello, 2003). From this point, methionine has various metabolic fates and participates in many important physiological processes including protein synthesis, DNA methylation, as well as other processes (Metayer et al., 2008).

2-hydroxy-4-methylthiobutanoic acid is absorbed and utilized by the body differently than methionine itself. Unlike methionine, HMTBA is absorbed primarily through diffusion as opposed to active transport (Dibner et al., 2004), although there is

some indication that a sodium-dependent mechanism is present as well (Marín-Venegas et al., 2007). After ingestion, HMTBA is absorbed along the entire length of the small intestine, although the majority of the absorption occurs in the proximal portion of the gastrointestinal tract (Richards et al., 2005). For the bird to utilize HMTBA post absorption, it must first be converted to L-methionine. This is accomplished by two enzymes that convert each respective isomer of HMTBA (D or L). The first is of these enzymes is L-2-hydroxy acid oxidase (L-HAOX) which is present mainly in hepatic and renal peroxisomes and converts the L isomer of HMTBA to L-methionine. The D isomer of HMTBA is converted to L-methionine by D-2-hydroxy acid dehydrogenase (D-HADH), which is present in the mitochondrial fraction of various tissues (Martín-Venegas et al., 2011). The conversion of HMTBA to methionine may be initiated as early as the absorption into the enterocytes (Marín-Venegas et al., 2006), but the conversion can take place in several tissues. While both sources of supplemental methionine are currently used in commercial practice, there have been conflicting reports with regards to the efficacy of HMTBA compared to DLM in an experimental setting. For example, Dibner et al. (2004) conducted an experiment in which birds were supplemented with equimolar amounts of either HMTBA or DLM and formulated diets to be deficient in methionine. Birds fed HMTBA tended to perform better than those birds which were fed diets supplemented with DLM. Another study conducted by Balnave and Oliva (1990) showed that birds supplemented with DLM displayed better feed efficiency than those supplemented with HMTBA under heat stress. However, a study conducted by Ribeiro et al. (2006) found no difference in growth performance for broilers supplemented with either compound. Some of A portion of the variation in performance seen between these two sources may depend

on several factors including dietary conditions (Vasquesz-Anon et al., 2006), arginine-to-lysine ratio (Balnave et al., 1999), level of DLM deficiency or supplementation (Elkin and Hester, 1983, Garlich et al., 1985, Saur et al., 2008), or level of cysteine (Thomas et al. 1983, Pillai et al., 2005).

Role of Methionine During Oxidative Stress

Although a necessary process for several functions in an aerobic organism, oxidative stress can have deleterious effects if it if there is prooxidant status within the body. Although the body employs various antioxidant defense systems to attenuate the potentially deleterious effects of free radicals, one of the most important antioxidants in the body of mammals and birds in glutathione. Glutathione (gamma-glutamyl-cysteinyl-glycine, GSH) is a tripeptide synthesized from glutamate, cysteine, and glycine in a series of two intracellular, ATP-dependent reactions. Glutathione is important in the context of cellular health and is known to play roles in oxidative and nitrosative stress as well as the detoxification of xenobiotics mediated through the glutathione-S-transferase system (Wu et al., 2004). It has been shown to be extremely important in maintaining the body's redox status and essential for the maintenance of cell health.

Methionine has an important role to play in maintaining GSH concentrations within the body. It is well-known that methionine can be catabolized to cysteine via the transmethylation-transsullfuration pathway. Using humans, rats, pigs, and chickens as a model, many studies have demonstrated that cysteine is indeed the limiting for GSH synthesis (Lyons et al., 2000; Jahoor et al., 1999; Chung et al., 1990). Increasing the

supply of cysteine or cysteine precursors via either oral or intravenous administration has been shown to enhance GSH synthesis and prevent GSH deficiency in both human and animals under different nutritional and pathological conditions (Townsend et al., 2003). Thus, it logical to suggest that methionine supplementation may contribute to the antioxidant status of an animal such as a broiler chicken. In this context, various studies have examined the effects of methionine supplementation and how it relates to the antioxidant status of broiler chickens, with special attention paid to GSH status. In a study conducted by Swennen et al. (2011), broiler diets were supplemented with varying levels of crude protein (CP) (18.3% or 23.2%) and were supplemented with methionine (0.25%) from either DLM or HMTBA. It was found that diets that were deficient in CP, but supplemented with HMTBA had higher levels of GSH and total GSH, suggesting HMTBA may provide a better antioxidant status. A similar study conducted by Willemsen et al. (2011) also examined the relationship between methionine source (DLM or HMTBA) and GSH status in heat stressed broiler chickens. This study also demonstrated source differences between the two methionine supplements, with HMTBA again yielding a more favorable antioxidant status than DLM. These experiment demonstrate the relation of methionine to antioxidant status via its link with GSH metabolism.

Effects on Embryonic Thermal Conditioning On Growth Performance

Epigenetic inheritance can be loosely defined as the inheritance of developmental variations that do not stem from differences in DNA sequence (Jablonka et al., 2009). Studies have demonstrated forms of epigenetic inheritance in various organisms including

rats, humans, and birds (Youngson and Whitelaw, 2008). One area of interest with regard to the practical utilization of epigenetic manipulation is the thermal conditioning of broiler chicks. Several studies have explored the impact of embryonic thermal conditioning in various poultry species, yielding mixed results on future performance. Research surrounding ETC has focused on changes in endocrinology, gut morphology, and growth performance (Barri et al, 2011, Uni et al., 2001, Yahav and Tzschentke, 2011). With regard to growth performance, studies generally show that ETC does not improve, but rather hampers the future growth performance of broilers. For example, a study conducted by Leksrisompong et al. (2007) showed that elevated incubation temperature significantly reduced body, heart, and intestinal weight on day of hatch. However, a second experiment within this study showed that ETC did not exert a significant effect on body weight at day of hatch. Another study conducted by Hulet et al. (2007) found that broilers incubated under higher than normal temperatures resulted in reduced body weight gain and increased FCR compared to birds incubated at a normal incubation temperature. In contrast, other research has shown that ETC leads to improved growth performance (Christensen et al., 1999). Other research has investigated other parameters related to gastrointestinal function as well as cell proliferation. For example, Barri et al. (2011) investigated morphological changes to the gastrointestinal tract during as well as nutrient transporter function in broiler chicks incubated at temperatures (39.6°C) from embryonic day 13 to 21. Results indicated that there was no difference in gene expression among various nutrient transporters including SGLT-1, GLUT-2, GLUT-1, or EAAT-3. However, there was increased PepT1 expression 10 days post-hatch compared to the control group that spent the entire incubation period at a temperature of 37.4°C. There were also morphological changes to

the gastrointestinal tract observed including shortened villi height and reduced crypt depth in the duodenum at 10 days post-hatch. These results implicate that ETC may actually exert negative effects on future performance. Conversely, other research has indicated some positive results with regard to muscle proliferation.

For example, Halevy et al., (2001) demonstrated that early post-hatch thermal conditioning (3 days of age for 24 hours) does seem to have a stimulatory effect on the number of myosatellite cells and satellite cell proliferation 5 days post-hatch. Another study conducted by Pietsun et al. (2009) demonstrated that ETC in broiler chicks from 16 to 18 days of development increased BWG at 25 and 35 days of age, as well as the pectoralis muscle as a percentage of body weight. This study also determined that ETC during this period of time increased several parameters associated with muscle hyperplasia including proliferating cell nuclear antigen (PCNA), insulin-like growth factor-1 (IGF-1), and thymidine incorporation into DNA of the examined muscle cells. Mixed performance in these studies may be due to a variety of reasons including the strain of bird investigated, timing of thermal conditioning, and the degree of environmental stress. Developmental factors that could be affecting the bird physiology include the development of the thermosensetive neurons located in the preoptic anterior hypothalamus (Yahav and Tzschentke, 2011).

Effects of Heat Stress on Digestibility

Dry matter digestibility of an animal diet correlates to the relative amount of a particular diet is being digested. Dietary constituents of feed are crucial components that

ensuring proper growth performance and health status of production animals. Decreased nutrient digestibility is often correlated with poor growth performance much like nutrient deficiency. This is also true with regards to ingredient digestibility in broiler chickens (Marsman et al., 1997; Li et al., 2007). Li et al. (2007) demonstrated the effects of feeding corn-soy diets supplemented containing chito-oligosaccharides (COS) from 1 to 42 days of age. Birds fed diets containing COS displayed better growth performance and increased digestibility of DM, CP, and energy compared to a control diet or a diet supplemented with chlorotetracycline, illustrating the relationship between digestibility and growth performance.

External influences such as psychological stress, disease stress, and environmental stress can exert an effect on the digestibility of individual feed components as well as feed dry matter as a whole. Bonnet et al. (1997) found that 4 week old broilers held under a constant 32° C for two weeks showed significantly lower digestibility of a corn-soy diet (70.2 at 22°C compared to 69.1 at 32° C). With regard to individual components of feed, there is considerable variability within the scientific literature. For example, various studies have shown slightly increased or non-significant changes in metabolizable energy (ME) during high environmental temperature (El Husseiny and Creger, 1980; Keshavarz and Fuller, 1980) in broiler chickens. Other studies have shown increased environmental temperatures decreased dietary ME content of diets fed to broiler chickens (Yamazaki and Zi-Yi, 1982), illustrating these inconsistencies. Plausibly, this may be related to variations in retention time as Hai et al. (2000) have shown that heat stress increases retention time in broiler chickens. While ME value of feed tends to decrease during a heat stress challenge, studies have generally demonstrated that protein and amino acid digestibility decrease in

hot environments (Wallis and Balnave, 1984). This is clearly detrimental to productive growth as the relationship between amino acid digestibility and even marginal deficiencies have been shown to affect growth performance. For example, Bregendahl et al. (2002) showed that broiler chicks fed diets marginally deficient in protein did not exhibit the same performance as birds fed adequate protein. Barnes et al. (2005) found that diets marginally deficient in sulfur amino acids negatively impacted growth performance in broiler chicks. Sebastian et al. (1997) demonstrated that using a microbial phytase increased apparent ileal digestibility of several amino acids, which was associated with increased growth performance in broiler chickens. Ghazi et al. (2002) found that broiler diets supplemented with a protease isolated from *Aspergillis* improved apparent ileal nitrogen digestibility along with growth performance from 7 to 28 days of age, again illustrating the link between increased protein digestibility and improved growth performance. Conversely, other studies have shown that protein deficiencies can reduce growth performance. When looking at digestive enzymes, Hai et al. (2000) found that market-age broilers exposed to temperatures of 32°C for a 6 day period showed decrease activities of trypsin and chymotrypsin, suggesting reduced capacity for protein digestibility. Thus, reduction in nutrient digestibility such as ME or amino acids are likely to have ramifications for the growth performance of broiler chickens during a heat stress challenge.

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CHAPTER 3

The effect of dietary methionine concentration and source on broiler performance and antioxidant status during acute and chronic heat stress

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A paper to be submitted to *Poultry Science*

Abstract

An experiment was conducted to determine the effects of varying source and concentration of digestible sulfur amino acids (DSAA) on growth performance and physiological response of broilers exposed to elevated temperatures. A 2 x 2 x 3 factorial arrangement was utilized to analyze performance data with methionine source; DL-methionine (DLM) or 2-hydroxy-4-methylthiobutanoic acid (HMTBA) at two DSAA concentrations (adequate: starter 0.94%, grower 0.84% or super-adequate: adequate concentrations +20% DSAA) and three environmental treatments: constant heat treatment (HT, 35°C), thermo-neutral (TN, 24°C), or thermo-neutral with pair-feeding commensurate to the feed intake of the heat stressed birds (TN-PF, 24°C). All other parameters were analyzed using a 2 x 2 x 2 factorial arrangement, not including the TN-PF in the analysis due to significantly different feed intakes between the HT and TN-PF treatments. Experimental diets were fed from d 1-27, while environmental treatments were applied from d 21-27. Blood and liver samples were taken at 6 h (acute) and on d 6 (chronic) of heat exposure to determine blood variables and hepatic antioxidant status. Heat treatment

lowered feed intake and body weight gain and increased the feed conversion ratio across all methionine sources and DSAA concentrations. Birds fed super-adequate DSAA had lower feed intake than those fed adequate DSAA. During the acute phase, HT lowered pCO_2, hematocrit, hemoglobin, and potassium concentrations and increased blood pH. Concentrations of oxidized glutathione in birds fed adequate DSAA during the acute phase were lower than birds fed super-adequate DSAA. Birds exposed to chronic HT had lower concentrations of total and reduced glutathione than in the TN groups. Hepatic concentrations of thiobarbituric acid reactive substances were lowest in birds fed DLM compared to HMTBA. No differences were found in hepatic glutathione peroxidase activity among treatments. These results indicate that heat stress significantly reduced performance, but generally did not affect lipid peroxidation or antioxidant status. Results indicated that source of methionine and concentration of DSAA did not consistently impact lipid peroxidation, antioxidant activity, or performance.

Key words: DL-methionine, 2-hydroxy-4-methylthiobutanoic acid, broiler, antioxidant, heat stress

Introduction

Heat stress has significant implications for broiler production and welfare, and thus the financial well-being of the poultry industry. A study conducted by St-Pierre et al. (2003) estimated that losses incurred by the U.S. broiler industry reach approximately $51.8 million annually. When subjected to elevated environmental temperatures, broilers exhibit a reduction in body weight gain (**BWG**), feed intake (**FI**), and an increased feed

conversion ratio (**FCR**) (Cooper and Washburn, 1998). A portion of the negative effects seen during hyperthermia may be related to the increased production of free radicals and reactive oxygen species (ROS). Reactive oxygen species are known to have deleterious effects on the constituents of biological tissues (protein, amino acids, lipids, and DNA), leading to cell damage and ultimately death (Fang et al., 2002). Elevated production of ROS during increased environmental temperatures may be due to disruption of the assemblies of the electron transport chain (Ando et. al., 1997). Reactive oxygen species production linked to heat stress has been reportedly associated with poor performance in meat-type chickens (Mujahid et al., 2005). Mujahid et al. (2007) reported that acute heat stress increases mitochondrial superoxide production in skeletal muscle and was linked with poor growth performance in 87 d old laying-type cockerels. Furthermore, ROS can contribute to the formation of other free radical species such as lipid hydroperoxides formed during lipid peroxidation (Benzie, 1996). Heat stress can also exacerbate the production of reactive compounds through ischemia and hypoxia (Hall et al., 1999). In order to cope with the over-production of ROS, the body employs a battery of antioxidant systems, in which methionine plays an important role.

Methionine is the often the first limiting amino acid in corn-soy poultry diets and plays a role in protein metabolism, immunity, and oxidative stress (Métayer et al., 2007; Zhang and Guo, 2008). Two commonly utilized sources of supplemental dietary methionine include crystalline DL-methionine (**DLM**) and 2-hydroxy-(4-methylthio) butanoic acid (**HMTBA**), a liquid methionine hydroxy analog. Routes of absorption differ between these two additives as DLM is absorbed via active transport in the gastrointestinal tract of broiler chickens whereas HMTBA is primarily absorbed via passive diffusion and

is subsequently converted to methionine within the body (Brachet and Puigserver, 1989). A possible mechanism through which methionine may mitigate the negative effects of heat stress is by indirectly serving as a substrate for glutathione production (Swennen et al., 2011). Glutathione (gamma-glutamyl-cysteinyl-glycine; reduced glutathione; **GSH**) is one of the body's primary antioxidant defense mechanisms and is the most common low-molecular weight thiol found in animal cells (Wu et al., 2004). Glutathione can react enzymatically and non-enzymatically with various free radicals and ROS, making it an essential component of the antioxidant defense system (Wu et al., 2004). Methionine conversion to cysteine is facilitated via the transmethylation and transsulfuration pathways, from which cysteine has various metabolic fates (Stipanuk et al., 2004). One such fate is incorporation of cysteine into glutathione, the production of which may be limited by cysteine availability (Chung et al., 1990). Given these factors, methionine and cysteine play a significant role in the body's antioxidant defense system.

The first objective of this experiment was to determine the effect of acute and chronic heat stress on growth performance and feed efficiency of birds fed two sources of methionine (DLM, HMTBA) at two different concentrations (adequate, super-adequate) of digestible sulfur amino acids (**DSAA**). The second objective was to determine the antioxidant status under these conditions by measuring hepatic concentrations of GSH, total GSH (**TGSH)** and oxidized glutathione (**GSSG**) as well as the activity of glutathione peroxidase (**GPx**), an important enzyme in protecting against oxidative damage. Concurrently, the concentration of thiobarbituric reactive substances (**TBARS**), an indicator of oxidative damage (Yagi, 1998), was measured to determine the degree of hepatic lipid peroxidation.

Materials and Methods

Experimental Design

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University prior to the start of the experiment. In total, eight hundred male Ross 308 broilers (Aviagen Inc., AL) were procured from a commercial hatchery (Longenecker's Hatchery, PA) at hatch and shipped via U.S. mail to the Iowa State University Poultry Research and Teaching Unit. Chicks were shipped in batches of four hundred as the entire experiment was conducted over two separate time periods. Chicks were brooded by experimental diet in floor pens (0.155 m^2 per bird) with pine shavings, nipple drinkers and pan feeders, providing *ad libitum* feed and water. Lamps brooders were provided for supplemental heat, keeping chicks at 35°C. Room temperature was held at 32°C and reduced by 2°C every week until transfer to environmental chambers at 21 d of age. Photoperiod provided during the 21 d rearing period was 23-h of light, 1-h of dark. From arrival to 14 d of age, birds were fed 1 of 4 experimental starter diets (one hundred birds per diet per time period) and were switched to experimental grower diets on d 14. Diets were formulated to meet or exceed nutrient requirements specified by the breeder (Aviagen Inc., AL) and were supplemented with either DLM (Rhodimet® NP 99) or HMTBA (Rhodimet® AT 88) from Adisseo SAS, France, formulated at two DSAA concentrations (adequate: starter 0.94%, grower 0.84% or superadequate: starter 1.13%, grower 1.00%; Table 3.1). At 21 d of age, remaining birds were sorted within their respective experimental diet by body weight, with care taken to minimize differences inweight distribution amongst all experimental units. After sorting, birds were allocated to 1 of 6 pens (0.15 m^2 per bird) within 8 environmental chambers that were kept in a

constant environment until initiation of the heat treatment. Environmental treatments were designated as heat treated (HT, 35° C; 44%Rh), thermoneutral (TN, 24° C; 75%Rh), or thermoneutral coupled with pair-feeding (TN-PF, 24° C; 76% Rh). Pair-feeding was achieved by measuring the feed intake of the HT group the first day of the experiment, when all treatments were fed ad-libitum. This amount plus an additional 10% was fed to the TN-PF group the next day to account for increasing growth requirements. Heat treated and thermo-neutral treatments were replicated in 9 pens while pair-fed birds were replicated in 6 pens due to limited space in the environmental chambers. Photoperiod in the environmental chambers was set at 24-h of light for the duration of the experiment. These procedures were followed for both runs of the experiment.

Data Collection

Chicks were monitored daily during the imposed heat stress period from 21 to 27 d of age. Body weights were recorded on a pen basis for each dietary treatment on d 1 and d 6 of the experiment; mortality was minimal (5 total mortalities from four separate treatment groups). For the HT and TN groups, feed intake was measured daily from d 21-27 by taking the initial weight of the feed and feeder and subtracting the difference in weight the next day. For all groups, FCR was then calculated by dividing the total feed consumed (g) per pen and dividing it by the total body weight gain (g) of the pen. At 6 h (on d 1) and 6 d of heat treatment, 1 bird per replicate pen from each respective treatment was euthanized by cervical dislocation. Blood samples were collected via an immediate post-mortem cardiac puncture, using a syringe coated in lithium heparin. Liver samples

were excised, flushed with distilled water, flash-frozen in liquid nitrogen, and stored at -80° C for further analysis.

Blood Sample Analysis

Blood collected was analyzed using the iSTAT® system in conjunction with a model GC8+ cartridge (Abbot Laboratories, IL). Analyzed blood characteristics included pH, partial pressure CO_2 (**pCO_2**), partial pressure of oxygen, HCO_3, base excess (**BE**), glucose, Na^+, K^+, total CO_2, ionized calcium, hematocrit (**Hct**), and hemoglobin (**Hb**).

Liver Sample Analysis

Quantification of hepatic TGSH and GSSG was performed using Glutathione Assay Kit (Cayman Chemical Company, MI) with units expressed as nmols/mg protein. Concentrations of GSH were calculated by multiplying the GSSG concentration by two and subtracting it from the total concentration of total glutathione (Willemsen et al., 2011). Hepatic GPx activity was determined using Glutathione Peroxidase Assay Kit (Cayman Chemical Company, Ann Arbor, MI), with units expressed as nmols/min/mg protein. Quantification of hepatic TBARS was determined using TBARS Assay Kit (Cayman Chemical Company, MI), with units expressed as nmols MDA/mg protein.

Statistical Analysis

For the performance data, statistical analysis was carried out using a 2 x 2 x 3 factorial arrangement of treatments. A 3-way ANOVA was performed using JMP (SAS Institute, Cary, NC) and Tukey's honestly significant difference test was utilized to separate significant least square means with the probability of type-I error put at $P \leq 0.05$. Blood and liver variables were analyzed as a 2 x 2 x 2 factorial arrangement due to the removal of the TN-PF group from the analysis in order to focus on difference between TN and HT environment.

Results and Discussion

Table 3.2 outlines treatment effects on BWG, FI, and FCR. There were no effects of methionine source or DSAA concentration on performance characteristics of broiler chicks exposed to acute or chronic heat stress, except for FI. Birds fed diets formulated to contain super-adequate levels of DSAA had a lower feed intake than those birds fed diets formulated to adequate concentrations. Ferguson et al. (1998) showed similar results in response to a high CP diet compared to one formulated to nutritional adequacy. Trends existed where birds fed DLM had an increased BWG ($P = 0.07$) and lower FCR ($P = 0.10$) compared to birds fed HMTBA. There was also a tendency for an interaction ($P = 0.07$) in which birds fed DLM in HT conditions had the lowest FI. The lack of significant response to methionine source or DSAA concentration seen in the remaining performance data (BWG, FCR) may have been due to the fact that all diets were formulated to meet dietary requirements for methionine and DSAA. Heat treatment significantly ($P < 0.01$) impacted

performance characteristics, lowering FI and BWG while increasing FCR compared to TN and TN-PF treatments. Impaired growth performance during heat stress is a well-demonstrated phenomenon and is in agreement with previous experiments (Geraert et al., 1996; Mujahid et al., 2009). The 10% increase in feed intake resulted in an approximately 6.7% over estimation of feed intake by the TN-PF group compared to the HT group. However, even though TN-PF birds had a 6.7% greater FI than HT birds, they experienced 15.7% greater BWG. This indicates that HT birds may have increased metabolic demands beyond what FI can account for alone.

Heat treatment during the acute phase lowered pCO_2, Hct, Hb, and K^+ levels while increasing blood pH ($P < 0.01$) compared to the TN group (Table 3.3). Mujahid et al. (2009) noted similar effects on broiler chickens at 34°C over a 6-h period, although the increase in pH observed in their case was not significant. It can be expected that blood pCO_2 was lower during the acute phase as birds displayed visibly increased panting during that period in order to facilitate evaporative cooling and can lead to an increase in blood pH (Richards, 1970). Changes in Hct and Hb concentrations may have been caused by hemodilution, a mechanism allowing for evaporative water loss without compromising plasma volume in the body (Borges et al., 2004). Lower K^+ concentrations observed in the HT group may have been due to increased excretion due to respiratory alkalosis. During respiratory alkalosis, there is reduced competition between H^+ and K^+ ions for urinary excretion, thus resulting in the increased excretion of K^+ (Borges et al., 2007). A trend was seen ($P \leq 0.06$) in which diets containing 20% above adequate DSAA during the acute phase had a lower pCO_2 compared to those fed adequate DSAA concentrations. Since the catabolism of cysteine derived from extra methionine in the diet can lead to increased

acidic end-products of metabolism (Bella et al., 1995), more blood CO_2 may have incorporated into bicarbonate to neutralize excess acid. However, bicarbonate levels did not significantly differ among the treatment groups, which may be due to the fact birds are efficient at reabsorption of filtered bicarbonate during acidosis (Toyomizu et al., 2005). During the chronic heat stress phase of the experiment, only K^+ differed significantly (P = 0.03) with HT lowering K^+ concentrations compared to the TN group (Table 3.4). The responses over the acute phase of the experiment and the lack of response over the chronic phase of the experiment suggests the ability of the broilers to adapt their blood chemistry to increased environmental temperatures over time.

There were significant differences in GSSG concentrations in the acute phase as well as differences in GSH and TGSH concentrations during the chronic phase (Table 3.5, Table 3.6). Concentrations of GSSG were significantly ($P < 0.01$) lower in birds receiving super-adequate DSAA than those receiving adequate DSAA in the acute phase. This may be related to a trend ($P = 0.06$) in which there were lower levels of TGSH in birds receiving super-adequate DSAA compared to birds fed adequate DSAA. A trend (P = 0.06) was also apparent where birds fed HMTBA had lower concentrations of GSSG compared to birds fed DLM. However, the GSH:GSSG ratio did not differ due to source. Another trend (P =0.07) was present in which birds fed super-adequate concentrations of DSAA possessed a higher GSH:GSSG ratio, related to the significantly lower level of GSSG observed in the birds fed super-adequate DSAA. During the chronic phase, the concentrations of both GSH and TGSH were higher ($P = 0.02$) in the HT group compared to the TN group. Heat shock is known to increase the synthesis of GSH in a variety of cells (Lu, 1999), which may have induced production of GSH, leading to higher a concentration

over the 6-d period. Trends were apparent during the chronic phase of environmental treatment with regard to the ratio of GSH to GSSG, an indicator of oxidative stress (Serru et al., 2001). A trend ($P = 0.06$) occurred in which birds fed HMTBA at adequate DSAA levels had a higher GSH:GSSG ratio compared to the other treatment groups. A tendency for a 3-way interaction ($P = 0.06$) was also present in which birds fed diets containing HMTBA formulated to adequate DSAA concentrations during heat stress had a higher GSH:GSSG ratio compared to other treatments. It has been previously reported that HMTBA provides more favorable hepatic GSH:GSSG ratio (Willemsen et al., 2011) and the data here indicate this trend as well. As with growth performance, the fact that diets were formulated to meet or exceed adequate DSAA concentrations in all treatment groups may have provided adequate substrate for GSH production and explain why no significant differences in the GSH:GSSG ratio were seen. This suggests that feeding the current concentration of DSAA may be sufficient to maintain glutathione status of broilers, even during heat stress.

Treatment effects on hepatic TBARS concentrations and GPx activity during acute and chronic heat exposure are outlined Tables 3.7 and 3.8, respectively. Birds fed DLM had lower TBARS concentrations ($P = 0.02$) compared to those fed HMTBA during the acute phase. Concurrently, a tendency for an interaction ($P = 0.07$) in which birds fed DLM in both environmental treatments (HT or TN) had lower TBARS concentrations in comparison to HMTBA was also observed. Although other studies have reported that HMTBA decreases plasma TBARS levels (Swennen et al., 2011; Willemsen et al., 2011), data here suggests that DLM better protected against hepatic lipid peroxidation. However, no significant differences were seen in the chronic phase of the experiment, suggesting

both compounds may provide similar protection against lipid peroxidation during a prolonged heat stress challenge.

The activity of GPx was not significantly different among any of the treatment groups during the acute phase despite the differences in GSH and GSSG concentrations among some of the treatments. A tendency towards a 3-way interaction was present (P = 0.08) during this phase in which DLM-fed groups generally possessed higher GPx activity. No significant treatment effects on GPx activity were seen during the chronic phase. Birds possess various antioxidant defense mechanisms to protect against free radicals and ROS such as the compound uric acid (Stinefelt et al., 2005). The lack of differences in GPx activity during the course of this experiment may have been due to contributions from other antioxidant mechanisms, thereby lowering the concentration of ROS available react with GPx.

In conclusion, increased environmental temperature had a significant impact on the growth performance of broiler chickens as illustrated by the decrease in BWG and FI as well increased FCR. Several of the blood characteristics measured in this experiment show that broilers were likely experiencing acute heat stress, but were able to adapt blood chemistry to chronic exposure. It appears that, in the acute phase, birds fed super-adequate concentrations of DSAA had lower GSSG concentrations, which may be due to a trend in which that treatment had lower TGSH concentrations. Both GSH and TGSH were lower in birds exposed to chronic HT. Super-adequate concentrations of DSAA as well as variable sources generally did not significantly impact lipid peroxidation, GPx activity, or exert a major influence on bird performance.

Acknowledgements

We would like to acknowledge Adisseo France SAS for funding this experiment through the Rhodimet Research Grant and kindly providing Rhodimet® NP 99 and Rhodimet® AT 88. We would also like to thank Lincoln Way Energy LLC for DDGS, Feed Energy Company for animal-vegetable blended fat, and ILC Resources for limestone. We recognize the care for the birds provided by W. Larson, J. Tjelta, W. Rogers, and R. Holbrooke of the Iowa State University Poultry Research and Teaching Unit. We would also like to thank A. Gabler, R. Murugesan, K. Nesheim, C. McDonald, and J. Tjelta of Iowa State University for their assistance in conducting this experiment.

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Table 3.1 Composition of starter and grower diets arranged by methionine source DL-met or HMTBA) and methionine concentration (adequate or super-adequate).

Diet	Starter (1-14 days)				Grower (14-28 days)			
Met Concentration	DLM		HMTBA		DLM		HMTBA	
Met Source	Adequate	+20%	Adequate	+20%	Adequate	+20%	Adequate	+20%
Ingredients	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Corn	55.00	54.78	54.96	54.71	59.24	59.05	59.20	58.99
DDGS	4.00	4.00	4.00	4.00	5.00	5.00	5.00	5.00
Meat/bone meal	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Soybean meal 48	31.94	31.97	31.95	31.98	25.81	25.83	25.81	25.84
AV Blend	2.17	2.16	2.17	2.16	3.61	3.60	3.61	3.60
Salt	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36
DL Methionine	0.31	0.51	-	-	0.26	0.43	-	-
HMTBA	0.-00	-	0.34	0.57	-	-	0.29	0.49
Lysine HCL	0.27	0.27	0.27	0.27	0.25	0.25	0.25	0.25
Threonine	0.06	0.06	0.06	0.06	0.04	0.04	0.04	0.05
Limestone	0.90	0.90	0.90	0.90	0.70	0.70	0.70	0.70
Dicalcium Phosphate	1.26	1.26	1.26	1.26	1.01	1.01	1.01	1.01
Choline Chloride	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin Premix[1]	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63
Nutrients	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Crude Protein	22.98	23.09	22.98	23.09	20.58	20.68	20.59	20.68
ME (kcal/kg)	3,025	3,025	3,025	3,025	3,150	3,150	3,150	3,150
Fat	5.37	5.35	5.37	5.35	6.98	6.96	6.97	6.96
Crude Fiber	2.75	2.74	2.75	2.74	2.71	2.71	2.71	2.7
Calcium	1.05	1.05	1.05	1.05	0.90	0.90	0.90	0.90
Available P	0.50	0.50	0.50	0.50	0.45	0.45	0.45	0.45
Digestible Met	0.61	0.80	0.61	0.80	0.54	0.7	0.54	0.7
Digestible Met + Cys	0.94	1.13	0.94	1.13	0.84	1.00	0.84	1.00
Digestible Lys	1.27	1.27	1.27	1.27	1.10	1.10	1.10	1.10

[1] Provided per kg of Diet: Selenium-250 µg; Vitamin A-8,250 IU; Vitamin D_3-2,750 IU; Vitamin A-17.9 IU; Menadione- 1.1 mg; Vitamin B_{12}-12 µg; Biotin-41 µg; Choline-447 mg; Folic acid-1.4 mg; Niacin-41.3 mg; Pantothenic acid-11 mg; Pyridoxine-1.1mg; Riboflavin-5.5 mg; Thiamine-1.4 mg; Iron-282 mg; Magnesium-125 mg; Manganese-275 mg; Zinc-275 mg; Copper-27.5 mg; Iodine-844 µg.

Table 3.2 Effects of methionine source (Source), digestible sulfur amino acid concentrations (Concentration), and environmental treatment (Environment) on feed intake, BW gain, and feed conversion ratio (FCR) in broiler chickens from 21-27 days of age.

Source	Concentration	Environment	Feed Intake	BW Gain	FCR
			(kg/pen)	(g/bird)	(g/g)
DLM			4.77	395.0	1.78
HMTBA			4.76	372.0	1.86
Pooled SEM			0.082	9.21	0.034
	A		4.88[a]	388.0	1.84
	+20		4.65[b]	379.0	1.80
	Pooled SEM		0.082	11.28	0.034
		TN	5.31[A]	435.0[A]	1.77[B]
		TN-PF	4.65[B]	383.0[B]	1.74[B]
		HT	4.34[C]	323.0[C]	1.96[A]
		Pooled SEM	0.101	11.27	0.041
		P-value			
Source			0.92	0.07	0.10
Concentration			0.05	0.46	0.45
Environment			<0.01	<0.01	<0.01
Source x Concentration			0.89	0.63	0.13
Source x Environment			0.07	0.78	0.46
Conc. x Environment			0.64	0.25	0.17
Source x Concentration x Environment			0.63	0.45	0.42

[A,B,C] Least square means within same column without a common superscript differ significantly, $P \leq 0.01$

DLM: DL Methionine, HMTBA: 2-hydroxy-4-methylthiobutanoic acid, A: Adequate methionine, +20%: Adequate methionine +20%, TN: thermoneutral (24°C), TN-PF: thermoneutral-pair fed (24°C), HT: heat treated (35° C), DSAA: digestible sulfur amino acids

Table 3.3 Effects of methionine source (Source), digestible sulfur amino acid concentrations (Concentration), and environmental treatment (Environment) on blood pH, pCO_2, K, Hct, and Hb during acute (6 h) heat stress in 21 day old broiler chickens.

Source	Concentration	Environment	pH	pCO_2 (mm Hg)	K (nmol/L)	Hct (%)	Hb (g/dL)	HCO_3
DLM			7.18	58.73	8.06	18.98	6.46	21.17
HMTBA			7.19	54.52	8.03	18.07	6.14	20.66
Pooled SEM			0.019	2.213	0.116	0.420	0.142	0.540
	A		7.17	59.62	7.94	18.56	6.31	21.60
	+20		7.20	53.63	8.15	18.50	6.29	20.23
	Pooled SEM		0.019	2.213	0.116	0.420	0.142	0.540
		TN	7.13^{B}	61.87^{A}	8.34^{A}	19.56^{A}	6.65^{A}	20.39
		HT	7.24^{A}	51.37^{B}	7.74^{B}	17.50^{B}	5.95^{B}	21.44
		Pooled SEM	0.019	2.213	0.116	0.420	0.142	0.540
			P-value					
Source			0.55	0.18	0.85	0.12	0.12	0.51
Concentration			0.25	0.06	0.21	0.92	0.93	0.08
Environment			<0.01	<0.01	<0.01	<0.01	<0.01	0.17
Source x Concentration			0.98	0.66	0.99	0.21	0.20	0.49
Source x Environment			0.61	0.43	0.83	0.75	0.75	0.78
Conc. x Environment			0.41	0.58	0.99	0.64	0.68	0.68
Source x Concentration x Environment			0.23	0.58	0.83	0.75	0.73	0.22

^{A,B}Least square means within same column without a common superscript differ significantly, $P \leq 0.01$

DLM: DL methionine, HMTBA: 2-hydroxy-4-methylthiobutanoic acid, A: adequate methionine, +20%: adequate methionine +20%, TN: thermoneutral (24°C), HT: heat treated (35°C), DSAA: digestible sulfur amino acids

Table 3.4 Effects of methionine source (Source), digestible sulfur amino acid concentration (Concentration), and environmental treatment (Environment) on blood pH, pCO_2, K, Hct, and Hb during chronic (6 d) heat stress in 27 day old broiler chickens.

Source	Concentration	Environment	pH	pCO_2 (mm Hg)	K (nmol/L)	Hct (%)	Hb (g/dL)	HCO_3
DLM			7.17	61.59	8.09	20.64	70.2	22.23
HMTBA			7.16	61.01	7.94	20.73	7.05	21.93
Pooled SEM			0.015	2.280	0.141	0.528	0.180	0.640
	A		7.19	60.51	8.08	21.14	7.19	22.73
	+20		7.15	62.10	7.95	20.23	6.88	21.43
	Pooled SEM		0.015	2.280	0.141	0.528	0.180	0.640
		TN	7.17	63.58	8.23	20.85	7.09	23.23
		HT	7.16	59.03	7.79	20.52	6.98	20.94
		Pooled SEM	0.015	2.280	0.141	0.528	0.180	0.640
				P-value				
Source			0.72	0.86	0.45	0.90	0.90	0.74
Concentration			0.07	0.62	0.52	0.23	0.23	0.15
Environment			0.68	0.16	0.03	0.67	0.68	0.01
Source x Concentration			0.41	0.14	0.86	0.07	0.07	0.34
Source x Environment			0.40	0.37	0.75	0.33	0.33	0.78
Concentration x Environment			0.81	0.60	0.93	0.67	0.68	0.47
Source x Concentration x Environment			0.66	0.60	0.10	0.33	0.35	0.64

[a,b] Least square means within same column without a common superscript differ significantly, $P \leq 0.05$

DLM: DL methionine, HMTBA: 2-hydroxy-4-methylthiobutanoic acid, A: Adequate methionine, +20%: Adequate methionine +20%, TN: thermoneutral (24°C), HT: heat treated (35°), DSAA: digestible sulfur amino acids

Table 3.5 Effects of methionine source (Source), digestible sulfur amino acid concentrations (Concentration), and environmental treatment (Environment) on total (TGSH) and reduced glutathione (GSH) as well as oxidized glutathione (GSSG) during acute (6 h) heat stress in 21 day old broiler chickens.

Source	Concentration	Environment	TGSH (nmols/mg protein)	GSH (nmols/mg protein)	GSSG (nmols/mg protein)	GSH:GSSG (nmols/mg protein)
DLM			53.95	34.95	9.59	4.75
HMTBA			47.22	30.44	7.65	4.96
Pooled SEM			3.919	3.088	0.724	0.642
	A		56.02	34.90	10.23^{A}	4.00
	+20		45.15	30.48	7.02^{B}	5.70
	Pooled SEM		3.919	3.088	0.724	0.642
		TN	52.77	34.74	8.95	4.85
		HT	48.40	30.65	8.29	4.85
		Pooled SEM	3.919	3.088	0.724	0.642
			P-value			
Source			0.23	0.31	0.06	0.82
Concentration			0.06	0.32	<0.01	0.07
Environment			0.43	0.35	0.52	0.99
Source x Concentration			0.18	0.20	0.29	0.40
Source x Environment			0.25	0.57	0.89	0.59
Conc. x Environment			0.97	0.68	0.89	0.44
Source x Concentration x Environment			0.53	0.96	0.53	0.67

[a,b] Least square means within a column without a common superscript differ significantly, $P \leq 0.05$

DLM: DL methionine, HMTBA: 2-hydroxy-4-methylthiobutanoic acid, A: Adequate methionine, +20%: Adequate methionine +20%, TN: thermoneutral (24°C), HT: heat treated (35°C), DSAA: digestible sulfur amino acids

Table 3.6 Effects of methionine source (Source), digestible sulfur amino acid concentrations (Concentration), and environmental treatment (Environment) on total (TGSH) and reduced glutathione (GSH) as well as oxidized glutathione (GSSG) during chronic (6 d) heat stress in 21 day old broiler chickens.

Source	Concentration	Environment	TGSH (nmols/mg protein)	GSH (nmols/mg protein)	GSSG (nmols/mg protein)	GSH:GSSG (nmols/mg protein)
DLM			54.63	31.69	11.76	3.07
HMTBA			53.63	36.32	11.36	3.72
Pooled SEM			3.530	2.737	1.091	0.290
	A		52.84	35.53	11.38	3.68
	+20		55.42	32.48	11.73	3.11
	Pooled SEM		3.530	2.737	1.091	0.290
		TN	48.34[b]	29.14[b]	10.90	3.09
		HT	59.92[a]	38.87[a]	12.21	3.69
		Pooled SEM	3.530	2.737	1.091	0.290
			P-value			
Source			0.84	0.24	0.80	0.12
Concentration			0.61	0.43	0.82	0.17
Environment			0.02	0.02	0.40	0.15
Source x Concentration			0.63	0.54	0.61	0.06
Source x Environment			0.65	0.62	0.19	0.21
Concentration x Environment			0.88	0.14	0.88	0.76
Source x Concentration x Environment			0.10	0.06	0.15	0.06

[a,b]Least square means within same column without a common superscript differ significantly, $P \leq 0.05$

DLM: DL methionine, HMTBA: 2-hydroxy-4-methylthiobutanoic acid, A: Adequate methionine, +20%: Adequate methionine +20%, TN: thermoneutral (24°C), HT: heat treated (35°C), DSAA: digestible sulfur amino acids

Table 3.7 Effects of methionine source (Source), digestible sulfur amino acid concentrations (Concentration), and environmental treatment (Environment) on thiobarbituric reactive substances (TBARS) concentration, and glutathione peroxidase (GPx) activity during acute (6 h) heat stress in 21 day old broiler chickens.

Source	Concentration	Environment	GPx (nmol/min/mg protein)	TBARS (nmol MDA/mg protein)
DLM			55.11	0.76[b]
HMTBA			40.55	1.18[a]
Pooled SEM			7.010	0.121
	A		47.87	0.84
	+20		47.79	1.10
	Pooled SEM		7.010	0.121
		TN	48.57	1.09
		HT	47.08	0.86
		Pooled SEM	7.010	0.122
		P-value		
Source			0.15	0.02
Concentration			0.99	0.14
Environment			0.88	0.19
Source x Concentration			0.48	0.52
Source x Environment			0.27	0.07
Conc. x Environment			0.61	0.23
Source x Concentration x Environment			0.08	0.36

[a,b]Least square means within same column without a common superscript differ significantly, $P \leq 0.05$

DLM: DL methionine, HMTBA: 2-hydroxy-4-methylthiobutanoic acid, A: Adequate methionine, +20%: Adequate methionine +20%, TN: thermoneutral (24°C), HT: heat treated (35°C), DSAA: digestible sulfur amino acids

Table 3.8 Effects of methionine source (Source), digestible sulfur amino acid concentrations (Concentration), and environmental treatment (Environment) on thiobarbituric reactive substances (TBARS) concentration, and glutathione peroxidase (GPx) activity during chronic (6 d) heat stress in 27 day old broiler chickens.

Source	Concentration	Environment	GPx (nmol/min/mg protein)	TBARS (nmol MDA/mg protein)
DLM			70.46	0.72
HMTBA			60.72	0.65
Pooled SEM			7.299	0.052
	A		68.64	0.70
	+20		62.54	0.68
	Pooled SEM		7.299	0.052
		TN	67.14	0.67
		HT	64.05	0.71
		Pooled SEM	7.299	0.052
DLM		TN	68.43	0.79
DLM		HT	72.48	0.66
HMTBA		TN	65.84	0.55
HMTBA		HT	55.61	0.76
		Pooled SEM	10.323	0.073
		P-value		
Source			0.35	0.35
Concentration			0.56	0.83
Environment			0.77	0.54
Source x Concentration			0.71	0.08
Source x Environment			0.49	0.03*****
Conc. x Environment			0.94	0.11
Source x Concentration x Environment			0.92	0.15

[a,b] Least square means within same column without a common superscript differ significantly, $P \leq 0.05$

DLM: DL methionine, HMTBA: 2-hydroxy-4-methylthiobutanoic acid, A: Adequate methionine, +20%: Adequate methionine +20%, TN: thermoneutral (24°C), HT: heat treated (35°C), DSAA: digestible sulfur amino acids

*****Means not significantly different using Tukey's honestly significant difference test to separate least square means

CHAPTER 4

The effect of embryonic thermal conditioning on ileal dry matter digestibility and growth performance in chickens

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A paper to be submitted to *Poultry Science*

Abstract

Two experiments were conducted to determine the effects of embryonic thermal conditioning on individual dry matter digestibility and growth performance of chickens exposed to 6 d of cyclic heat stress. In Experiment 1, advanced intercross line (AIL) were exposed to normal incubation temperature (NI) of 37.8°C from embryonic day -21 to 0 or an elevated incubation temperature (EI) treatment of 37.8°C from embryonic day -21 to -12, increased to 39.5°C from embryonic day -12 to -4, then back to 37.8°C until hatch. Chicks were reared in floor pens with access to corn-soy diets. On day 17, birds were transferred to floor pens in environmentally controlled chambers set at a thermoneutral environment (TN) at 23.8°C. Individual body weights were recorded on day 21 and day 28 of the experiment. One group of birds was subjected to cyclical heat stress (HS, 35° C) for a 7 hour period until 28 days of age. Ileal samples were collected for analysis of dry matter digestibility, with the ileum being defined as the section of intestine from Meckel's diverticulum to the ileal-cecal junction. All procedures followed in Experiment 1 were

followed in Experiment 2, except all eggs were incubated at 37.8°C. During Experiment 1, there were no significant treatment effects BWG from 21 to 28 d of age. Ileal dry matter digestibility at 28 d of age was higher in EI birds compared to NI birds. An interaction occurred in which birds reared at elevated incubation temperatures in a thermoneutral environment the highest ileal dry matter digestibility of 93.8, with birds incubated under normal temperatures in a thermoneutral environment having the lowest (91.4). In Experiment 2, environment significantly influenced BWG, with the HS group gaining significantly more weight than the TN group. No significant effect was seen on digestibility. No significant correlations were found between ileal dry matter digestibility and BWG in experiments one or two. It can be concluded from this experiment that ETC may exert an effect on ileal dry matter digestibility, but not directly on growth performance.

Keywords: Embryonic thermal conditioning, epigenetic inheritance, dry matter digestibility

Introduction

Heat stress is a significant issue affecting the broiler industry, both from the standpoints of animal welfare as well as the performance, ultimately reducing the profitability of producers. Heat stress is known to be associated with poor growth performance in broilers and is known in some cases to reduce energy, amino acid, and dry matter digestibility (Bonnet et al., 1997; Cooper and Washburn, 1998, Wallis and Balnave, 1984). In the United States alone, it is estimated that heat stress contributes to losses of

approximately $51 million to the U.S. broiler industry (St. Pierre et al., 2003). Several experiments have explored the effects of heat stress on poultry species after a period of increased embryonic thermal conditioning (**ETC**). These experiments have examined the effects of ETC on several variables including the development of the nervous system (Tzschentke et al., 2004), growth performance (Hulet et al., 2007), as well as the acquisition of heat tolerance (Moraes et al., 2003). Although results have been mixed, several studies have shown that the effects of thermally conditioning of poultry improved heat tolerance during a heat stress challenge (Piestun et al, 2008). Methods of manipulating poultry development to aid in the acquisition of heat tolerance may be of use in hot climates. However, little information is available as with regards to its subsequent effects on feed digestibility, particularly during periods of heat stress. Heat stress has been shown to decrease the feed digestibility of corn-soy broiler diets (Bonnet et al., 1997). However, there are conflicting data present in the literature concerning the digestibility of individual nutrients and feed ingredients. Various experiments have reported a small increase or non-significant changes in ME during high environmental temperature (El Husseiny and Creger, 1980; Keshavarz and Fuller, 1980). Other experiments have reported increased environmental temperatures decreased dietary ME content (Yamazaki and Zi-Yi, 1982). Other studies have found that increased environmental temperatures have little effect on amino acid digestibility in both broilers and layers (Wallis and Balnave, 1984; Koelkebeck et al., 1998). Given the variability in the literature in the digestibility of nutrients, further investigation in warranted as to the effects of embryonic conditioning on nutrient digestibility.

While the previously mentioned experiments have explored many variables with regard to the effects of ETC, literature is limited as to the effects of ETC on subsequent progeny. While research has shown that epigenetic modifications can be achieved in early thermal conditioning, both posthatch and prehatch, little information is available with regard to effects on subsequent generations. Evidence of similar performance of subsequent generations or lack thereof would indicate the presence of epigenetic modifications and whether they can be inherited by offspring. Epigenetic inheritance has been shown in other species such as rats, humans, and birds (Youngston and Whitelaw, 2008). Therefore, the objective of this experiment was to characterize the effect of ETC on individual feed dry matter digestibility and growth performance of chickens during cyclical heat stress. A second objective was to further characterize the epigenetic response to thermal conditioning by determining the individual dry matter digestibility and growth performance of the offspring of parents incubated at either normal (**NI**) or elevated (**EI**) incubation temperatures.

Materials and Methods

Experiment 1

Experimental Design

This experiment was approved by the Iowa State University (ISU) Institutional Animal Care and Use Committee. Three hundred forty nine fertile eggs of advanced intercross line chickens (broiler x Fayoumi; **AIL**) were placed in incubators at the Iowa State University Poultry Research and Teaching Unit. Eggs were incubated at either a

normal incubation temperature (NI) of 37.8°C from embryonic day -21 to 0 (day of hatch) or held at 37.8°C from embryonic day -21 to -12 and subsequently held at an elevated incubation temperature (EI) of 39.5°C from -12 to -4, then back to 37.8°C until hatch. This period of manipulation is similar to other authors attempting to affect the hypothalamus-hypophysis-thyroid axis in an attempt to affect heat tolerance in broiler chickens (Piestun et al., 2008). On day of hatch, 349 birds were placed in floor pens containing pine shavings, nipple drinkers, and pan feeders from 1 d of age until 17 d of age. Corn-soy starter and grower diets were fed based on requirements outlined by the National Research Council (1994) recommendations for poultry. Bird were kept on a photoperiod of 24 h of light during the rearing period. Lamps brooders were provided for supplemental heat while room temperature was kept at 32° C at day-of-age and reduced by 2°C every week until transfer to environmental chambers at 17 d of age. At day 17 of age, birds were moved into floor pens contained within environmentally controlled chambers, maintained at a temperature of 25°C. Individual bird body weights were taken at 21 days of age. At 22 d of age, birds were divided into two groups, one held at a thermoneutral temperature (TN) of 25°C or subjected to a cyclical heat stress treatment (HT) at a temperature of 35°C maintained for a period of 7 hours per day until 28 days of age (7 total days of elevated temperature). During the cycle, temperatures reached 35°C within the first hour and were maintained until hour 7 in which temperatures were lowered back to 25°C. At hatch, birds that were to be placed in either HT or TN environments had similar body weights (28.2 g in HT group; 29.9 g in TN group). By 21 days of age, body weights were 243.9 in the HT treatment and 238.2 in the TN treatment. Bird numbers per individual treatment were as follows: 11 birds in the EI-HS group, 14 birds in the EI-TN group, 312 birds in the NI-HT

group, and 12 birds in the NI-TN group. The large number of birds in the NI-HT treatment was to allow for genetic mapping in a separate experiment. Birds were fed a corn-soy based diet based on NRC recommendations (1994). At 28 d of age, birds were euthanized via injection of sodium pentobarbital into the jugular vein according to the dosage recommendation of the manufacturer (Fort Dodge Animal Health, Overland Park, KS). Following euthanization, body weights were recorded and ileal samples were collected, with the ileum being defined as the intestinal portion from Meckel's diverticulum to the ileal-cecal junction. Ileal samples were expressed into bags, sealed and subsequently stored at -20°C for further analysis of dry matter digestibility.

Determination of Dry Matter Digestibility

Ileal and feed samples were dried for 24 hours in a drying oven overnight at 110° C for determination of dry matter. Titanium dioxide was used as an inert marker in both feed and fecal samples. Titanium concentration was analyzed using the method described in Leone (1973) with modifications. Determination of dry matter digestibility was determined using the following equation:

$$\left[\frac{\left(\%\ Diet\ DM - \frac{\%\ Fecal\ DM\ X\ \%\ Diet\ Ti}{\%\ Fecal\ Ti}\right)}{\%\ Diet\ DM}\right] \text{X } 100$$

Experiment 2

Experimental Design

This experiment utilized 167 fertile eggs from AIL birds. The experimental design and procedures were conducted in an identical fashion as Experiment 1, except that in this case all embryos were incubated at a temperature of 37.8°C. Birds used in this experiment were the progeny of parents previously incubated in Experiment 1 at either 37.8°C or 39.5°C (parents of these progeny were not utilized in Experiment 1 and only used for breeding purposes). Therefore, the treatment groups in this experiment consisted of groups whose parents were incubated at a normal parental temperature (PN) of 37.8°C or progeny whose parents were incubated at an elevated parental temperature (PE) of 39.5°C from embryonic day -12 to -4. As in Experiment 1, these groups were further divided into treatments consisting of a TN or HS environment. As in Experiment 1, birds that were to be placed in either HT or TN environments had similar starting body weights (28.88 g in HT group; 28.91 g in TN group).). By 21 days of age, body weights were 329.7 in the HT treatment and 321.0 in the TN treatment. Bird numbers per individual treatment were as follows: 26 birds in the PH-HS group, 8 in the PH-TN group, 125 in the PN-HS group, and 8 birds in the PN-TN group. The same parameters (body weight gain, dry matter digestibility) and collection procedures were used as in Experiment 1.

Statistical Analysis

Statistical analysis was carried out using a 2 x 2 factorial arrangement, with incubation temperature and environmental temperature as the two factors. Data were

analyzed using PROC MIXED of SAS (SAS Institute, Cary, NC) with least squared means (LSM) to separate means and student's t-test (α= 0.05; t=1.98698) was used to separate significant LSM with the probability of type-I error put at $p \leq 0.05$. Differences among treatments were separated using Tukey's honestly significant difference test. A Pearson Correlation between ileal dry matter digestibility and BWG was performed using PROC CORR of the SAS system (SAS Institute, Cary, NC)

Results and Discussion

In the first experiment, there were no effects of any treatment groups on BWG (Table 4.2). A large body of research shows that high environmental temperatures are detrimental to the growth performance of broiler chickens (Cooper and Washburn, 1998; Geraert et al., 1996; Mujahid et al., 2009). This may have been due to the strain of bird used in this experiment as they are smaller in body size compared to broiler strains typically used in the industry. Berrong and Washburn (1998) conducted an experiment in which the performance of broilers and a traditional poultry breed were compared during a 3 week heat stress period. The smaller-framed traditional breed showed a less severe depression in growth performance compared to the broiler strain. The study also found that the traditional breed had a lower body temperature than the broiler strain across all temperatures tested (21, 32, and 38°C), offering a possible explanation for performance differences.

At 28 d of age, a main effect of incubation on feed dry matter digestibility was present in which birds in the EI treatment had a high digestibility than those in the NI

treatment (Table 4.2). These results suggest that embryonic thermal conditioning altered feed digestion. Previous reports have shown that periods of heat stress increase the digestibility of feed in poultry species (Tur et al., 1985). Barri et al (2011) demonstrated that embryonic thermal conditioning decreased PEPT1 expression post-treatment, but did not affect various other digestive enzymes. This suggests that another mechanism may be at work that is altering the digestion the digestion of feed. One possible explanation may be variations in triiodothyroxine (T3) production related to varying incubation temperatures. Moraes et al. (2002) demonstrated that broilers incubated at higher than normal temperatures (39° C for 2 h daily from embryonic d 13 to 17) displayed a larger drop in T3 than those birds incubated at normal temperatures. These studies may point to effects associated with hypothyroidism, which has been demonstrated to decrease the passage time of digesta in young broiler chickens (Tur et al., 1986). This may be partly responsible for the increased digestibility seen in birds at EI since an increase retention time may allow more time for the digestion of feed as has been demonstrated in swine (Kim et al. 2007). This indicates that elevated incubation temperature may have altered the gastrointestinal function of the bird.

A significant interaction also occurred in which EI birds raised in the thermoneutral environment had the highest dry matter digestibility while NI birds raised in a thermoneutral environment had the lowest dry matter digestibility (Table 4.2). Within the NI group, HS significantly increased digestibility compared to NI birds in the TN group. Uni et al. (2001) found that heat treatment 3 days post-hatch decreased circulating T3 levels, although lower villus volume 24 h post-treatment as well as reduced enterocyte proliferation 48 h post-treatment was found. However, enterocyte proliferation and

digestive enzyme activity increased in the subsequent days. As with the effects of incubation alone, these results may be related to the decreased production of thyroid hormones during high ambient temperature. The fact that the birds in the EI-TN treatment were able to maintain a similar digestibility to those in the EI-HT treatment may again demonstrate that, even in a thermoneutral environment, elevated incubation exerts a positive effect on digestibility. However, these results did not translate into increased growth performance as there were no significant effects of incubation on growth performance and the correlation between growth performance and digestibility (P=0.27), nor were there significant correlations between these variables amongst individual treatment groups within both experiments (data not shown).

During Experiment 2, there was a significant effect of environment on growth performance (Table 4.3), with the HT group having a higher BWG than the TN group. As previously mentioned, heat stress is known to exert a significant effect on growth performance. It is surprising to see that not only did increased temperature not impair growth performance, but actually improved it. The smaller body frame size of the AIL birds in this study may have contributed to this result compared to what would be commercially utilized in the broiler industry. This factor in conjunction with a possibly less severe heat stress challenge in the form of cyclic heat stress may have not hampered bird performance and allow them to experience higher growth than the TN birds. Some researches have demonstrated that the effects of cyclic heat stress are not as severe as chronic heat stress (Azad et al., 2003; Osman et al., 1989). A final factor may be that the HT group had a numerically higher body weight compared to the TN group (329.7 vs.

321.0), which may partially explain the increased body weight gain in the HT group during the experiment.

Parental incubation condition had no effect on BWG in Experiment 2. This is not surprising due to the fact that there was no significant effect of incubation on BWG in Experiment 1. Even though other studies have shown reduced organ weights and growth performance due to embryonic thermal conditioning (Hulet et al., 2007; Leksrisompong et al., 2007), neither experiment in this present study saw these effects of incubation on BWG. Dissimilar conditions such as incubation time and strain may be responsible for the lack of response growth performance shown with incubation. There was no significant treatment effects on dry matter digestibility (Table 4.3). Although there was a significant effect of HT on digestibility in the previous experiment, there exists some variation in the literature with regard to nutrient digestibility during heat stress. Bonnet et al. (1997) found that 4 week old broiler held under a constant 32° C for two weeks showed significantly lower digestibility (70.2 at 22°C compared to 69.1 at 32° C). Amino acid digestibilities (Wallis and Balnave, 1984) as well as dietary ME (El Husseiny and Creger, 1980; Keshavarz and Fuller, 1980; Yamazaki and Zi-Yi, 1982) have been shown to decrease during heat stress. A trend (P = 0.10) was present in which the PE group showed a higher digestibility than birds from the PN group, possibly related to epigenetic inheritance. However, this must be further characterized and confirmed utilizing molecular techniques. As with Experiment 1, no correlation was seen between growth performance and ileal dry mater digestibility (P= 0.83).

Based on the results of these experiments, ETC had no significant effects on growth performance, but did affect ileal dry matter digestibility in Experiment 1. There

was no effects of environment on BWG or ileal dry matter digestibility. In Experiment 2, no significant effects of parental incubation were seen on BWG or digestibility. The birds in the HS group had a significantly higher BWG than the TN group, possible due to size-related differences of the strain used compared to conventional broilers. It can be concluded from this experiment that ETC may exert an effect on ileal dry matter digestibility, but not directly on growth performance.

Acknowledgements

The authors thank the staff of the Iowa State University Poultry Research and Teaching Unit for assistance in animal care, including W. Larson, J. Tjelta, W. Rodgers, and R. Holbrook. The authors also thank D. Coble, A. Bjorkquist, S. Onteru, C. McDonald, R. Murugesan, N. Nachtrieb, L.Bramer, P.Liu, K. Nesheim, and M. Kaiser for their assistance in conducting this experiment. This work was supported by USDA-NIFA-AFRI Climate Change award 2011-67003-30228.

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Table 4.1-Diet Formulations (Experiments 1 and 2)

Diet Phase	**Starter**	**Grower**
Ingredients	**%**	**%**
Corn	57.54	62.82
Soybean meal 48	32.70	28.75
AV Blend	5.24	4.38
Salt	0.46	0.33
DL Methionine	0.23	0.09
Limestone	1.20	1.28
Dicalcium Phosphate	1.91	1.38
Choline Chloride	0.10	0.10
Vitamin Premix[1]	0.63	0.63
TI	-	0.25
Nutrients	**(%)**	**(%)**
Crude Protein	22.27	20.58
ME (kcal/kg)	3,200	3,150
Fat	7.86	6.98
Crude Fiber	2.49	2.71
Calcium	1.00	0.90
Available P	0.45	0.45
Digestible Met	0.50	0.54
Digestible Met + Cys	0.81	0.84
Digestible Lys	1.01	1.10

[1]Provided per kg of Diet: Selenium-250 µg; Vitamin A-8,250 IU; Vitamin D_3-2,750 IU; Vitamin A-17.9 IU; Menadione- 1.1 mg; Vitamin B_{12}-12 µg; Biotin-41 µg; Choline-447 mg; Folic acid-1.4 mg; Niacin-41.3 mg; Pantothenic acid-11 mg; Pyridoxine-1.1mg; Riboflavin-5.5 mg; Thiamine-1.4 mg; Iron-282 mg; Magnesium-125 mg; Manganese-275 mg; Zinc-275 mg; Copper-27.5 mg; Iodine-844 µg.

Table 4.2-Treatment effects on body weight gain and dry matter digestibility of corn-soy diets in Experiment 1

Incubation Temperature	Environmental Treatment	DM % Digestibility	BWG (g/bird)
NI		92.29[b]	145.41
EI		93.43[a]	146.45
Pooled SEM		0.31	7.347
	HT	93.14	147.84
	TN	92.58	144.02
	Pooled SEM	0.31	7.567
NI			
	HT	93.21[A]	151.00
	TN	91.38[B]	141.90
EI			
	HT	93.07[AB]	144.69
	TN	93.78[A]	146.14
	Pooled SEM	0.435	9.347
	----------*p value*----------		
Incubation		0.01	0.89
Environment		0.20	0.66
Incubation x Environment		0.004	0.50

[a,b] Least square means within same column without a common superscript differ significantly, $P \leq 0.05$

[A,B] Least square means within same column without a common superscript differ significantly, $P \leq 0.01$

NI: Normal Incubation Temperature (37.8°C), EI: Elevated Incubation Temperature (39.5°C from d -12 to -4), HT: Heat Treated (35°C 7-h daily), TN: Thermoneutral (25°C)

Table 4.3-Treatment effects on body weight gain and dry matter digestibility of corn-soy diets in Experiment 2

Parental Incubation	Environmental Treatment	% Digestibility	BWG (g/bird)
PN		86.59	136.92[b]
PE		88.72	138.93[a]
Pooled SEM		3.285	12.400
	HS	89.21	146.23
	TN	86.09	129.63
	Pooled SEM	3.297	12.446
----------***p value***----------			
Incubation		0.10	0.79
Environment		0.21	0.04
Incubation x Environment		0.81	0.17

[a,b] Least square means within same column without a common superscript differ significantly, $P \leq 0.05$

NI: Normal Incubation Temperature (37.8°C), EI: Elevated Incubation Temperature (39.5°C from d -12 to -4), HT: Heat Treated (35°C 7-h daily), TN: Thermoneutral (25°C)

CHAPTER 5

GENERAL CONCLUSIONS

The objective of this thesis was to evaluate potential strategies for mitigating the detrimental effects of heat stress in chickens. Two different approaches were utilized: The first explored the effects of supplemental digestible sulfur amino acids (DSAA) from two different methionine sources on growth performance, lipid peroxidation, antioxidant status, and blood chemistry variables of broilers during both acute (6 hours) and chronic (6 days) heat stress. The second strategy examined the effects of embryonic thermal conditioning (ETC) on growth performance and feed dry matter digestibility in broiler x Fayoumi crosses under a heat stress challenge and furthermore set out to determine if these effects would be observed in subsequent progeny.

In the first experiment, feed intake (FI), body weight gain (BWG) and feed conversion ratio were negatively affected by chronic heat stress. This was illustrated not only by the poor growth performance seen in the heat treated (HT) birds compared to birds held at a thermo-nuetral (TN) temperature, but also the HT group compared to the thermo-neutral group coupled with pair-feeding (TN-PF). Although there was an overestimation of the FI needed for the TN-PF group, this overestimation was determined not be enough of a difference in FI account for all the performance lost in the HT group. This is consistent with several studies that have also indicated such results in broiler (Balnave and Brake, 2001; Cooper and Washburn, 1998; Gareart et al., 1996, Mujahid et al., 2005; Willemsen et al., 2011). Treatment differences existed in which DLM fed birds had a lower TBARS

concentration than birds fed HMTBA during HT. However, hepatic TBARS concentrations did not increase due to HT in either the acute or chronic phase. This is unusual since similar environmental stress has been shown to increase TBARS in the liver (Lin et al., 2006). It is difficult to determine what the cause of this may be, but it could be related to contributions from other antioxidant defense mechanisms preventing extensive lipid peroxidation to occur.

There were no differences in enzymatic antioxidant capacity as measured by glutathione peroxidase (GPx). This was somewhat of a surprising result considering that many studies have shown that heat stress increases the activity of antioxidant enzymes, especially during an acute heat stress challenge. Altan et al. (2003) found that acute heat stress (3 hours at 38°C) increased activities of not only GPx, but also catalase and superoxide dismutase, both of which are important enzymes in the antioxidant defense system in eukaryotic organisms. Interestingly, this experiment examined both Ross and Cobb strains of broilers and found that this increase in antioxidant activity was only found to be in the Ross birds. Although the birds used in the first experiment of this thesis were of Ross stock, this still demonstrates the possibility of variable enzyme responses during heat stress. Furthermore, the results found in Altan et al. (2003) were observed at an even higher (38°C) temperature than used in the first experiment of this thesis (35°C). This gives credence to the distinct possibly that heat stress imposed may not have been severe enough to impose oxidative stress on the broilers. This would explain lack of treatment differences in the apparent activity of hepatic GPx as well as hepatic lipid peroxidation.

However, one could still argue that GPx activity would be expected to increase on the basis of substrate utilization as the synthesis off GSH synthetic enzymes are

upregulated during heat shock (Wu et al., 2004). Theoretically, this should increase the availability of GSH which acts as a cofactor for GPx, therefore increasing its enzymatic activity. In fact total concentrations of glutathione were significantly increased during the acute phase in the first experiment, which is consistent with increases in GSH production during heat shock. However, the presence of this peptide does not conclusively indicate the production of free radicals or ROS. Glutathione plays an important role in numerous cellular events (Wu et al., 2004) that do not necessarily involve the detoxification of free radical and ROS. Therefore, caution should be taken when basing scientific conclusions simply on concentrations of reduced and oxidized glutathione.

With regard to the chronic phase of HT, lack of differential activity in antioxidant enzymes during heat stress is not a phenomenon that is unheard of. Willemsen et al. (2011) found that exposing broilers to 32°C from 2 to 6 weeks of age did not affect plasma superoxide dismutase activity, nor plasma uric acid levels. Azad et al. (2009) found that two weeks of heat exposure, whether chronic or cyclic, did not have an effect on GPx activity in broilers. Lin et al. (2006) found that there were no significant differences in superoxide dismutase activity in 33 day old broilers exposed to 32° C for 6 hours, another case supporting a lack of acute response. These data lead one to the conclusion that chronic heat stress induces some adaptor mechanisms such as increased transcription or translation activity of antioxidants.

The acute phase demonstrated classic signs of heats stress with regard to blood variables as seen in other studies (Mujahid et al., 2009), but lack of changes over the chronic phase point to adaptor mechanism within the birds physiology, whether it be through renal acidification to combat alkalosis or increased absorption if minerals to keep

the bird in a homeostatic state. Taken together, the results from the first experiment imply that, while environment may have affected growth performance, neither methionine source nor DSAA concentration exerted significant effects. This suggests that supplementing DSAA beyond what is required for nutritional adequacy is not an effective means to combat the effect of heat stress. Several factors could have contributed to these results as heat stress is known to have a multitude of effects on broiler growth performance and physiology. It is likely that the multiple negative effects of heat stress were more than could be combated by supplemental DSAA alone. In addition to free radical production, heat stress has been shown to decrease the rate of protein synthesis (Lin et al., 2006), which apparently cannot be restored by increasing the level of protein in the diet (Temim et al., 2000). Likely, this could play a major role in the reduction in body weight of chronically heat stressed broilers, especially with regard to lean tissue accretion. This is accompanied by a reduction in blood flow to the visceral tissues as much as 44% (Bottje and Harrison, 1984), which may reduce nutrient transport, further contributing to poor performance. Effects of heat stress may be present at the gastrointestinal level as well. Heat stress has been shown to compromise intestinal epithelial integrity and decrease macrophage activity, which is associated with poor broiler performance (Quinteiro-Filho, et al. 2010). Compromised gastrointestinal integrity may lead to bacterial translocation, of which the effects can be compounded by a compromised immune system.

While super-adequate methionine supplementation does not seem improve performance or antioxidant status, some follow up approaches could be undertaken to further isolate the effects of free radicals during heat stress to better understand their contribution to the negative effects of heat stress. This may include the measurement of

other antioxidant defense mechanisms such as uric acid, superoxide dismutase, and catalase. Direct measurement of free radical production using paramagnetic electron spin trapping in various tissues may also provide a more detailed picture as to the true production of free radicals during bouts of heat stress and correlate the production of these compounds to antioxidant activity within the body.

The second portion of the thesis research examined the effects of embryonic thermal conditioning (ETC) on bodyweight gain (BWG) and feed dry matter digestibility in advanced interline crosses (commercial broiler x Fayoumi; AIC). During the second experiment, there were no treatment effects on BWG, but there were observed effects on dry matter digestibility. Overall, digestibility was increased by elevated incubation temperature (EI) in both HT and TN conditions, while those birds at a normal incubation temperature (NI) only displayed increased digestibility in HT conditions, but not TN conditions. It was surprising to see that a cyclic heat stress did not impair growth performance in this experiment. Several experiments have demonstrated the negative impact of cyclic heat stress on growth performance in various species of poultry (Balnave and Brake, 2001; Balnave and Oliva, 1990; Yahav et al., 2007). However, others contest that cyclic heat stress is a less sever challenge compared to chronic heat stress (Daghir, 2008). Some studies have demonstrated that the drop in growth performance is actually greater in chickens than are exposed to chronic heat stress rather than acute heat stress (Osman et al., 1989). Azad et al. (2006) conducted a study in which broilers were exposed to chronic or cyclic heat stress from 2 to 4 weeks of age. Broilers held at constant heat stress had significantly lower feed intake, bodyweight gain, and feed efficiency compared to birds held in cyclic heat stress. This may explain partially explain the lack of negative

effects that cyclical heat stress had on the AIC in this case. It was interesting to note that birds in the EI treatment displayed increased digestibility in both environmental conditions. This may be related to differences in triiodothyroxine (T3) production seen during ETC. Moreas et al. (2003) conducted their experiment in which broiler embryos were thermally conditioned during a similar period of time as the second experiment of this thesis and found reduced T3 production. Plausibly related, the effects of hypothyroidism have been shown to decrease digesta passage time in broilers (Tur et al. 1987). However, there was no associated performance difference correlated with increased digestibility. While this result was seemingly counterintuitive, feed dry matter digestibility does not accurately portray all the dietary component that are absorbed by the bird. Individual component digestibility may have been a limiting factor to growth, perhaps cancelling out any benefit from overall digestibility of the diet as a whole. Another confounding factor is the fact that that a microflora population is present throughout the gastrointestinal tract which may utilized dietary nutrients. In that case, the increased digestibility due to an increase in retention time may have simply been a function of increased microbial-related disappearance in the gastrointestinal tract. Furthermore, variations in individual feed intake were not accounted for in this experiment, which likely exerted a significant influence on performance. That is, if the HT group was able to maintain similar feed intakes to that of the TN group, it is plausible to assume that significant difference in BWG would not be detected.

During the third and final experiment within this thesis, there was a significant effect of environment on growth performance in experiment 3, with the HT group having a higher BWG than the TN group. Again, this is a somewhat perplexing result due to the

documented negative effects of heat stress as discussed above. As stated in Chapter 4, the smaller body size of the AIC birds compared to commercial birds or the fact that cyclic heat stress may be less severe may partially explain why no negative effect of HT on BWG were observed. However, this does not fully elucidate the mechanism by which birds were able to actually able to demonstrate improved performance in the HT environment. The negative consequences of heat stress, whether cyclic or chronic or conducted for varying time periods, has been consistently shown to decrease performance compared to poultry held at generally thermoneutral conditions. Although it cannot be confirmed in this experiment, perhaps there was a thermal conditioning effect from the environmental treatment itself. Birds in the HT group in both the second and third experiments were exposed to 35° C for 7 hours from 21 to 27 days of age. Similar experimental conditions have yielded birds which are more resistant to heat stress. A study conducted by Givisiez et al. (1999) observed increases in hepatic heat shock protein 70 (Hsp 70), a protein that assists with the proper protein folding during heat stress, as well as preventing apoptotic events at the cellular level (Kregal, 2002) was associated with increased heat tolerance. This same study found that bodyweight gain in birds exposed to cyclic heat stress did not have statistically different final body weight than birds reared in a thermoneutral environment. As with the second experiment, variations in individual feed intake may be related to BWG differences if in fact birds in the HT group were able to consume more feed than the TN group. With regard to incubation conditions, there was no effect of either EI or NI and BWG or feed dry matter digestibility. Piestun et al. (2008) found that thermal manipulations during a similar incubations phase, which coincided with the development of the hypothalamus-hypophysis-thyroid axis, significantly improved thermotolerance in

broiler chickens. However, this experiment initiated thermal conditioning two days earlier than this experiment and concluded it a day sooner. Perhaps the slight deviation from this period did not exert as great of an impact on the hypothalamus-hypophysis-thyroid axis, precluding significant effects. However, given the fact that there were treatment interactions affecting dry matter digestibility in the second experiment, but none in the third experiment, there may be a lack of trans-generational epigenetic inheritance present within the third experiment's progeny. However, given that only minimal phenotypic data were utilized for this study, this is purely speculative as there may be epigenetic modifications present, but none that are able to manifest themselves as a visible phenotypic response. Further verification using molecular techniques to investigate epigenetic mechanisms such as chromatin marking or RNA-mediated epigenetic inheritance. Incubation effects on BWG in the second experiment as well as BWG and digestibility in the third experiment may have been more pronounced during a chronic heat stress challenge.

In conclusion, heat stress remains a significant issue affecting broiler performance, welfare, and the profitability of producers. The first experiment demonstrated the negative consequences of heat stress, even beyond reduced feed consumption, but failed to demonstrate any significant effect of methionine source or concentration on growth performance. The second experiment demonstrated that digestibility was affected by incubation, but BWG was not. The third and final experiment demonstrated no significant effects of incubation on either response variable. Results in the first experiment suggest that DLM and HMTBA are equally effective methionine supplements, even though some research has demonstrated HMTBA to be more effective during heat stress challenges

(Willemsen et al., 2011). Although ETC has been demonstrated to improve heat tolerance in some cases, results here indicate no tangible benefit to improving performance during a heat stress challenge, even though there may be mild improvements in digestibility. Based on these observations, neither one of these strategies should be considered an effective means to improve growth performance during a heat stress challenge.

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