Effects of Methionine Supplementation on Broilers Raised under High Stocking Density and High Ambient Temperature

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Master of Science

by
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ABSTRACT

Two studies were conducted to investigate effects of supplementing 100 or 130% of the required digestible methionine to corn-soybean meal-based grower and finisher diets (1) as DL-methionine (DL-MET) for broilers housed in a higher density and (2) as either DL-MET or 2-hydroxy-4-(methylthio)butanoate (HMTBA) for broilers exposed to a high ambient temperature. In the first study, the high density impaired ($P < 0.05$) growth performance in both phases, decreased ($P < 0.05$) liver and adipose tissue fatty acid concentrations, and increased ($P < 0.05$) glutathione (GSH) in all assayed tissues except for the liver of the growers. The 130% DL-MET supplementation decreased ($P < 0.05$) feed intake of the finishers as well as the finisher breast and thigh malondialdehyde (MDA) levels. DL-MET elevated ($P < 0.05$) GSH in the grower thigh and fatty acid concentrations in the finisher liver. The hepatic expressions of heat shock protein 90 (HSP90) was decreased ($P < 0.05$) by the extra methionine supplementation. In the second study, the 130% methionine supplementations of both forms enhanced ($P < 0.05$) hepatic GSH concentrations of the growers and ferric reducing ability of plasma (FRAP) of the finishers. The DL-MET-fed growers had greater ($P < 0.05\%$) muscle GSH and hepatic unsaturated fatty acid concentrations than those fed HMTBA. Expression of inflammation-related genes in the liver of finishers was affected ($P < 0.05$) by interaction effects of the methionine form and concentration. In summary, extra methionine supplementation showed moderate beneficial effects on tissue antioxidant status and growth performance of broilers under environmental stresses.

BIOGRAPHICAL SKETCH

Guanchen Liu was born in Fuzhou, China on October 25, 1994. After finishing high school, Guanchen went to China Agricultural University (CAU) in Beijing and majored in Animal Science. Then he participated in the CAU "2+2" exchange program and transferred to Cornell University, and majored in Animal Science for two years. As an undergraduate student at Cornell, Guanchen joined Dr. Xingen Lei's lab and started participating in poultry nutrition-related experiments. After graduating from Cornell, Guanchen went on to pursue a Master's degree in Animal Nutrition under the supervision of Dr. Lei. His master research projects were to investigate effects of methionine supplementations in diets of broilers on their responses to high stocking density stress and high ambient temperature until August 2019.

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LIST OF ABBREVIATION

AKP, Alkaline phosphatase

AKT, Protein kinase B

ALT, Alanine aminotransferase

DL-MET, DL-methionine

FRAP, Ferric reducing ability of plasma

GR, glutathione reductase

GSH, glutathione

GSSG, glutathione disulfide

GST, glutathione S-transferase

GPX, glutathione peroxidase

HMTBA, 2-hydroxy-4-(methylthio)butanoate

HSP70, Heat shock protein 70

HSP90, Heat shock protein 90

IL-6, Interlukin-6

IL-10, Interlukin-10

JNK, c-Jun N-terminal kinase

MDA, malondialdehyde

MUFA, Monounsaturated fatty acid

NEFA, Non-esterified fatty acid

PIP, Inorganic phosphate

PUFA, Polyunsaturated fatty acid

P38MAPK, P38 mitogen-activated protein kinases

ROS, Reactive oxygen species

SFA, Saturated fatty acid

SOD, superoxide dismutase

TC, Total cholesterol

TG, Triglyceride

TNFα, Tumor necrosis factor alpha

TRAP, Tartrate-resistant acid phosphatase,

WHC, Water holding capacity

CHAPTER ONE

Introduction

1.1 Broiler industry

Chicken meat is the second most consumed meat in the world following pork. Approximately 95.5 million tons of chicken meat was produced worldwide in 2018[1]. The U.S is the largest producer of chicken meat globally, produced 19.3 million tons of chicken compared to Brazil and China, ranked the second and the third, produced 13.6 and 11.7 million tons respectively in 2018[1]. In the U.S, the broiler industry has created more than 1.2 million job opportunities and 95 billion dollars was expended on purchasing broiler related products in 2018[2]. Although the broiler industry is well developed and successful at generating large profits every year, it still faces many challenges and how to solve the negative impacts on the broilers brought by stress conditions like high stocking density and high ambient temperature is one of them.

1.2 Stress conditions and oxidative stress

The world population is approximately 7.7 billion as of 2019 and this number is excepted to reach 8.6 billion by 2030[3]. The total food production output needs to be increased in order to meet the massive nutritional demand for this rapidly increasing global population. This demand will be higher in those developing countries with higher increasing rates of population. In order to increase the production of

chicken meat to meet the requirement of projected meat consumption without impairing the efficiency of space utilization, modern broiler houses are designed to fit thousands of broilers at once. This results in high stocking density [body mass (kg) or the number of birds per unit of housing space (m^2 or ft^2) broilers]. Broilers raised in such conditions are thought to be susceptible to stocking density stress which disturbs the normal physiological equilibrium or homeostasis and results in depressed immune responese[4-5], impaired growth performance, and increased rates of the mortality[6-8].

Currently, the southern part of the U.S is responsible for most of the chicken meat production and the top 5 broiler producing states in the U.S. are Georgia, Alabama, Arkansas, North Carolina, and Mississippi[9]. These states tend to have high average temperatures especially during summer months. Heat stress is the major concern for the performance and welfare of animals raised in high ambient temperature. A negative balance between the net amount of energy flowing from the animal to the surrounding environment and the amount of heat produced by the animals is thought to cause heat stress in the animals[10]. Broilers under heat stress will show symptoms such as increased respiration rates (panting), increased wings spreading, and lethargy. Birds raised under high ambient temperatures also have compromised immune responses, decreased growth performance, increased mortality rates, and impaired meat quality[10-11]. As global temperatures keep rising due to global warming, the prevalence of these problems could be worsened.

Reactive oxidative species (ROS) are the byproduct of numerous physiological and biochemical processes in broilers. Under normal conditions, intrinsic antioxidant defenses in animals can detoxify the generated ROS to maintain the redox homeostasis[12]. While under stressed conditions such as high stocking density and high ambient temperature, the formation of ROS would be elevated and the balance between ROS production and intrinsic antioxidant defenses would be disturbed. The excessive ROS can then adversely modify cellular proteins, lipids, and DNA structures leading to oxidative stress[13,14-16] which impairs performance and wellbeing of broilers. Therefore, targeting methods to decrease excessive ROS to reduce oxidative stress could serve as a potential solution to relief the adverse effects caused by the stress conditions.

1.3 Methionine and its antioxidant capacity

Antioxidants such as vitamin E, carotenoids, and sulfur-containing amino acids (methionine and cysteine) are known for their abilities to scavenge ROS generated under stressed conditions[17-18]. Supplementing these antioxidants to diets of broilers under high stocking density and high temperature could be a potential solution to reduce the damage caused by oxidative stress. Methionine is an essential sulfur-containing amino acid required for tissue growth and protein synthesis. As the first-limiting amino acid to broiler birds fed commercial corn and soybean meal diets, methionine is also a potent antioxidant. Methionine exerts its antioxidant capacity in animals mainly in two ways. Firstly, methionine residues in proteins can directly scavenge ROS. As a sulfur containing amino acid, methionine residues on the surface of proteins is readily oxidized. By scavenging ROS and being

oxidized into methionine sulfoxide, methionine can protect other critical residues in proteins from oxidation, therefore, maintain their integrity and function. Furthermore, methionine sulfoxide can be reduced by intrinsic enzymes methionine sulfoxide reductases back to methionine to regain its antioxidant capacity[19-21] **(Figure 1)**. Secondly, methionine can be converted into homocysteine in the one-carbon cycle in the liver[22]. Thereafter, homocysteine can be converted into cysteine through transsulfuration[22]. Cysteine is not only a potent antioxidant itself, but also a precursor for the synthesis of glutathione (GSH)[23-25], an intrinsic antioxidant and a potent scavenger of ROS[26-27] **(Figure 2)**.

Synthetic DL-methionine (DL-MET) and 2-hydroxy-4-(methylthio)butanoate (HMTBA) are two commonly-used methionine supplements in animal diets. DL-MET is the combination of both D- and L-isomers of methionine. HMTBA is the hydroxy analog of methionine, with the amino group in methionine replaced with a hydroxyl group. Both the D-MET and HMTBA compounds need to be converted into L-methionine in a two-step process which mainly happens in the liver and also in some other tissues such as the small intestine and kidney of the broilers[28-29]. HMTBA is also known to have a lower bioavailability compared to DL-methionine, which is thought to be due to differences in absorption, conversion, and utilization[30-32]. Previous studies showed that both forms of methionine supplementations in the diets can improve the growth performance and antioxidant status of broilers. Chen and colleagues showed that supplemental methionine improved the growth performance and oxidative status of broiler chicks[33], Rama Rao and Swain's groups also found that supplementing methionine in the broiler diets would enhance their growth performance and immune responses[34-35].

Tang and colleagues demonstrated that HMTBA supplementation in broiler diets could improve the intestinal functions and oxidative status of the broilers[36]. Pontin et al found that supplementing HMTBA in broilers diets could enhance their growth performance[37].

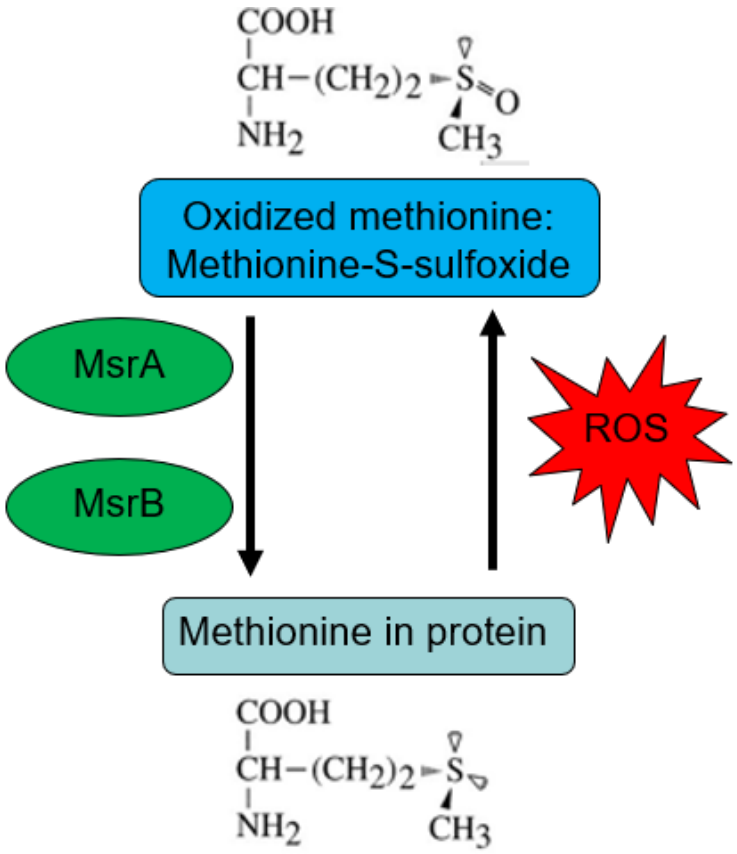

Figure 1. Antioxidant capacity of methionine by scavenging ROS. Adapted from "Antioxidant effects of sulfur-containing amino acids," by Atmaca, G., Yonsei Med J 2004, 45 (5), 776-88. MsrA: Methionine sulfoxide reductases A; MsrB: Methionine sulfoxide reductases B. Methionine residues in proteins is readily oxidized by ROS into methionine-S-sulfoxide protecting the critical residues in the proteins from oxidation. Oxidized methionine can recover its antioxidant capacity since methionine-S-sulfoxide can be reduced by intrinsic enzymes MsrA or MsrB back to methionine.

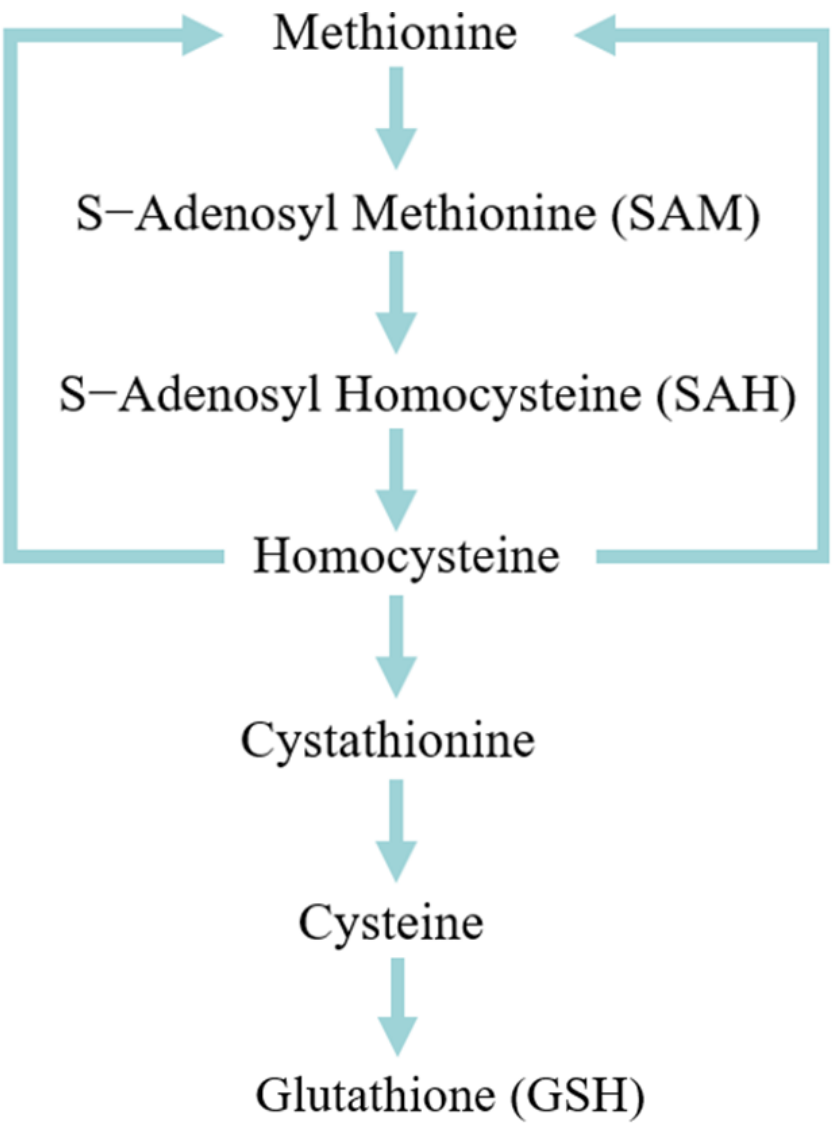

Figure 2. Antioxidant capacity of methionine by involving in one carbon cycle. Adapted from "The methionine-homocysteine cycle and its effects on cognitive diseases," by Miller, A. L., Altern Med Rev 2003, 8 (1), 7-19. Methionine can be converted into homocysteine in the one-carbon cycle in the liver. Thereafter, homocysteine can be converted into cysteine through transsulfuration. Cysteine is sulfur containing amino acid with potent antioxidant capacity, it is also a precursor for the synthesis of GSH.

1.4 Rationale for the proposed analyses

The activities of alanine aminotransferase (ALT) in the plasma is considered an indicator of liver damage in the broilers, and elevated levels of plasma ALT is usually related to impaired liver function[38]. The plasma level of alkaline phosphatase (AKP) also serves as a biomarker of broiler health where elevated AKP is frequently associated with hepatobiliary disease and impaired bone health[39]. The concentration of inorganic phosphate (PIP) is utilized as a measurement of both kidney and bone health. Increased activities of plasma PIP are usually related to impaired kidney functions and bone health[40]. Uric acid is the end product of nitrogen metabolism and also an important antioxidant in the broilers, therefore its plasma concentration is considered both a biomarker for tubular function and oxidative status[41]. For the above described reasons, these biomarkers were measured to assess the health status of broilers in the studies. Plasma glucose and lipid profiles including triglyceride (TG), total cholesterol (TC), and non-esterified fatty acid (NEFA) are also commonly tested biomarkers for animal health status, their concentrations were also measured in the current studies.

Breast and thigh are the key products of broiler meat production. Muscle tissues with a higher lean composition and less fat content are reported to be more desirable[42]. Therefore, the lipid profiles were analyzed in the breast and thigh tissues. The lipid profiles were also measured in the liver tissue to investigate the effects of methionine supplementation and the stress conditions on fat depositions in the liver. Fatty acids especially long chain unsaturated fatty acids are susceptible to oxidation[43].

Furthermore, previous studies showed that in chickens under stress, such as heat stress or stress induced by corticosterone exposure, more of the consumed energy was stored in fat instead of being used for other purposes[44-47]. It is also relevant to note that lipid synthesis and activation of fatty acid synthase could relate to proinflammatory status[48], while breakdown of fatty acids might be related to anti-inflammatory phenotypes[48]. Therefore, measuring the fatty acid profiles in the tissues of the broilers could be an indicator of oxidative stress.

GSH is an intrinsic antioxidant and a potent scavenger of ROS[26-27] in the broilers. It can also be synthesized from methionine in the one carbon cycle as described above. Glutathione peroxidase (GPx) is an intrinsic antioxidant enzyme, it protects the animals from oxidative stress by catalyzing GSH into glutathione disulfide (GSSG) to scavenge free radicals[49-50]. Similarly, glutathione S-transferase (GST) also catalyzes GSH resulting in detoxification of ROS[51] and glutathione reductase (GR) restores GSH level in the body by reducing its oxidized form, GSSG, back to GSH to maintain the antioxidant capacity of GSH[52]. Superoxide dismutase (SOD) is another important antioxidant enzyme that defenses the system against oxidative stress by catalyzing ROS into molecular oxygen and hydrogen peroxide[53-55]. Due to the antioxidant capacities of GSH and the antioxidant enzymes, measuring the concentration of GSH and activities of these enzymes could well represent the antioxidant status of the broilers. The ferric reducing ability of plasma (FRAP) is a measurement of the total antioxidant capacity of the plasma, its level was also analyzed in these studies to access the antioxidant status of the broilers. Malondialdehyde (MDA) is the byproduct of lipid peroxidations[56] and protein carbonyl is produced

when proteins are oxidized[57], therefore, their concentrations were measured as the biomarkers of oxidative stress in the studies. The levels of serum amyloid A and corticosterone were tested to indicate inflammatory status of the broilers because both their levels would increase in response to inflammation[58-59].

Previous researches described tumor necrosis factor alpha (TNFα) as a proinflammatory cytokine that can induce ROS generation and inflammation[60-62]. The abundance of interleukin-6 (IL-6) as a pro-inflammatory cytokine is also elevated during inflammation[63-64]. The elevated IL-6 and the excessive ROS up-regulate mitogen-activated protein kinases (MAPK) such as P38MAPK and c-Jun N-terminal kinase (JNK) and subsequently induce protein kinase B (AKT) production to protect cells from oxidative injury or death[60, 65-66]. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that protects the animals from inflammation with the potential to inhibit ROS generation[67-68]. Its level as well as the levels of heat shock proteins (HSP70, HSP90) were shown to be elevated under oxidative stress[67-72]. The mRNA levels of these inflammation-response cytokines were therefore tested to indicate the oxidative and inflammatory status of the broilers.

Dressing percentage and meat to bone ratio are commonly recorded to evaluate the carcass quality of the broiler. Water holding capacity and pH were measured to determine meat quality of the breast and thigh. The double compression texture profile analysis to simulate the mouth biting action was also performed on breast and thigh. Hardness, springiness, and chewiness were measured by the analysis. Hardness

represents how firm the tested product is and springiness measures how well a product physically springs back after it has been deformed. Chewiness is calculated from hardness and springiness representing the mouthfeel sensation of chewing the product, breast or thigh, in the studies. Woody breast and white stripling are two muscle myopathies that are commonly seen in broiler breast muscle; therefore, their severities were measured in the studies. Bone strengths were tested on the tibia of the broilers to assess the effects of stress conditions and methionine supplementation on bone health of the broilers.

1.5 Research objectives

Dietary supplemental methionine has been shown to improve antioxidant status, anti-inflammatory response, growth performance, and wellbeing of broilers. However, few studies have determined if those benefits could be enhanced by elevating the methionine supplementation and(or) vary with its chemical form in broilers exposed to high stocking density or high ambient temperature. Therefore, these two studies were conducted to test a working hypothesis that elevating supplemental DL-MET and HMTBA from the 100% to the 130% of the required digestible methionine concentrations into corn-soybean meal-based grower and finisher diets for broilers would help the animals cope with the high stocking density or high ambient temperature-induced metabolic stress. The objectives of the first study were to compare if the two concentrations of supplemental DL-MET exerted similar or different effects on: 1) growth performance, meat quality, feather coverage, and bone strength of broilers; and the 2)

antioxidant status, health indicators, inflammation-related gene expression, and lipid and fatty acid profiles in different tissues of broilers. The objectives of the second study were to compare if the two forms and concentrations of supplemental methionine exerted similar or different effects on 1) growth performance, meat quality, feather coverage, and bone strength of broilers; and 2) antioxidant status, health indicators, inflammation-related gene expression, and lipid and fatty acid profiles in several tissues of broilers.

CHAPTER TWO

Supplemental methionine in broilers subjected to high stocking density.[1]

2.1 Abstract

To study the effects of supplemental methionine in the grower and finisher diets for broilers under high stocking density, 560 day-old Cornish Cross cockerels were divided into four groups and raised under two stocking densities (1.0 and 1.3 chick/ft^2). Two groups in each density were fed diets supplemented with 100 or 130% required methionine as DL-MET. The high methionine elevated ($P<0.05$) GSH concentrations in the thigh at both ages. The high density elevated ($P < 0.05$) GSH concentrations in the plasma, breast, and thigh of growers, but decreased ($P < 0.05$) its concentrations in the liver of the growers and thigh of the finishers. Interaction effects ($P < 0.05$) between dietary methionine and stocking density were found on activities of the antioxidant enzyme GST in the liver of the growers and breast, thigh, and adipose tissue of the finishers. The interaction effect was also found on activities of GPX and SOD in the thigh of the growers. The high methionine decreased ($P < 0.05$) the hepatic expression of heat shock protein HSP90 and MDA concentrations in the thigh and breast of the finishers. High stocking density decreased ($P < 0.05$) body weight of growers and finishers, and total average daily gain. Increased stocking density and supplemental DL-MET both decreased ($P < 0.05$) feed intake and

[1] Guanchen Liu, Andrew D. Magnuson, Tao Sun, Samar A. Tolba, Xi Lin, Rose Whelan, and X. G. Lei. Supplemental methionine affects antioxidant status, fatty acid profiles, and growth performance of broilers raised at a high stocking density. Current Developments in Nutrition 2019, To be submitted.

improved ($P < 0.05$) feed efficiency of the growers and finishers. In conclusion, extra methionine supplementation as DL-MET mitigated effects caused by the high stocking density and improved antioxidant status of the broilers.

2.2 Introduction

Modern broiler houses are designed to raise flocks of these animals at relatively high densities to save space and increase revenues. However, broilers raised under these conditions may suffer from stocking density stress. As the space per bird decreases, growth performance and immune status of birds are adversely affected [4-8].

ROS are byproduct of numerous biochemical reactions in animals which occur more frequently when animals are under stress. It is well established that environmental stress, including crowding, can cause excessive ROS production and lead to oxidative stress in broilers. Oxidative stress impairs the health and growth performance of broilers[73-74]. Dietary antioxidants, compounds which can neutralize ROS and reduce oxidative stress, are known to improve animal performance during said stress conditions [18, 33, 43, 75]. Thus, supplementing antioxidant nutrients in broiler diets may ameliorate the adverse effects brought by the elevated stocking density.

Sulfur containing amino acids such as cysteine and methionine are known for their capability to scavenge ROS [19]. Methionine is the first limiting amino acid in chicken diets which is not only

important for protein synthesis, but also plays an important role in antioxidation. Supplementing dietary methionine beyond growth requirements has been shown to improve antioxidant status [33] and immune function [34-35] of growing broilers. The sulfur in methionine is readily oxidized into sulfoxide under oxidative conditions, which enables methionine to scavenge ROS during oxidative stress, protecting other amino acids with essential functions [19-21]. Methionine is also directly involved in the one -carbon cycle [22]. In the cycle, methionine can be converted into cysteine which is also a sulfur containing amino acid and the precursor of GSH, a potent antioxidant [22]. Therefore, methionine not only possesses a potent antioxidant capacity itself but also supports other antioxidant systems.

Although previous studies indicate that the impair on health and performance of broilers under high stocking density was caused by oxidative stress and methionine possesses potent antioxidant capacity. It has been seldom studied if extra supplemental methionine in broiler diets can improve their performance of broilers under high stocking density. Therefore, we conducted this study aimed to test the hypothesis that 30% extra DL-MET supplementation in the grower and finisher diets of the broilers could improve the antioxidant status, health status, and improve the growth performance of birds under high stocking density. Our objectives were to compare if the two concentrations of supplemental DL-MET exerted similar or different effects on: 1) growth performance, health status, meat quality, feather coverage, and bone strength; 2) lipid and fatty acid profiles, antioxidant status, and expressions of inflammation-related genes.

2.3 Materials and methods

2.3.1 Animals, diets, and management

The protocol was approved by the Cornell University Institutional Animal Care and Use Committee. The experiment was conducted at the LARTU, Cornell University, Ithaca, NY. A total of 560 Cornish Cross cockerels purchased from Moyer's Chicks, Quakertown, PA were raised in 1 m by 1 m floor-pens in environmentally-controlled rooms with 2:22 h dark-light cycles with free access to water and feed. The birds were randomly divided into 4 treatment groups (10 pens/group) in a 2x2 factorial arrangement with stocking density and DL-MET supplementation level as the main effects. Birds were raised in one of two stocking densities, a normal density of 9 birds per pen equal to 1 bird per ft^2, or at a high density treatment of 12 birds per pen equal to 1.3 birds per ft^2. All the birds were fed the same corn and soybean-meal based diet during the starter period (day 0 – 10). During the grower (day 11 - 22) and finisher (day 23 – 42) periods, two experimental diets were fed to the birds with two supplementation levels of DL-MET. The supplemented DL-MET product was >99% pure (MetAMINO ® , Evonik Industries, Essen, Germany). In one treatment methionine was supplemented at 2.90 and 2.60 g DL-MET/kg of diets in the grower and finisher diets, respectively, to meet 100% of the methionine requirement of broilers based on AMINOChick® 2.0 (Evonik Nutrition & Care GmbH, Germany). The second dietary treatment methionine was supplemented at 3.77 and 3.38 g/kg of DL-MET/kg of diet in the grower and finisher diets, respectively, to provide 130% of the methionine requirement. The diets

were formulated to meet all other nutrient requirements of the broilers and nutrient compositions of the diets are shown in **Table. 1**.

2.3.2 Sample collection

Body weight and feed intake were recorded weekly to evaluate growth performance. Mortality was recorded daily. At the end of grower and finisher periods, CO_2 asphyxiation followed by cervical dislocation was used to euthanize the birds (n=80) for sample collection. Blood was collected from the hearts and the plasma was obtained by centrifugation at 12000 g for 15 min at 4°C (Beckman GS-6R centrifuge, Brea, CA). A small portion of the liver was collected and immediately frozen in liquid nitrogen for gene expression measurement. The rest of the liver, adipose, breast and thigh muscle were dissected and kept on dry ice and later stored at -20°C for biochemical and meat quality analysis. Tibias were collected from each bird (n=80) after removal of the muscle, tendon, and ligament and were subsequently stored at -20°C for bone strength test.

2.3.3 Plasma health indicators

The activities of ALT, AKP in the plasma and the concentrations of PIP, glucose, TC, TG, NEFA, and uric acid were analyzed following methods described in previous studies[76-77].

2.3.4 Tissue lipid and fatty acid profiles

The lipid profiles (TC, TG, and NEFA) were measured in the liver, adipose tissue, breast, and thigh with methods in previous studies[76-77]. The fatty acids were extracted and methylated using the method

developed by Christie[78]. The gas chromatography-mass spectrometry (model HP 5890 A with an HP 5970 series mass-selective ion-monitoring detector, Hewlett-Packard, Palo Alto, CA) with the internal standard of tritridecanoin was used to analyze fatty acid profiles.

2.3.5 Tissue and plasma antioxidant status

Concentrations of GSH, GSSG in the plasma, liver, breast and thigh and concentrations of MDA in the liver, adipose, breast and thigh were assayed using methods adapted from previous studies[76-77]. Benzie and Strain's method was used to determine the FRAP level[79]. Concentrations of protein carbonyl in the liver were determined using a method developed by Oliver et al[80]. Commercial kits (Cayman Chemical, Ann Arbor, MI) were used to measure plasma corticosterone and serum amyloid A. Activities of GPx, GST, GR, and SOD were measured in the breast, thigh, liver and adipose tissue using methods adapted from previous studies[81-84].

2.3.6 Quantitative real-time PCR

Abundances of IL-6, IL-10, TNFα, HSP70, HSP90, AKT, P38MAPK, and JNK mRNA in the liver were determined. Primers used for these tested genes are listed in **Table 2.** Total mRNA was isolated and purified from the liver using TRIzol Reagent (Life Technologies, Carlsbad, CA) following the established method[85]. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY) was used to do the mRNA reverse transcription. The Real-time qPCR (7900 HT; Applied Biosystems) and the $2^{-\text{delta delta Ct }(\Delta\Delta Ct)}$ equation[86] were then used to quantify the mRNA levels.

2.3.7 Meat quality, bone strength, breast muscle myopathy, and feather coverage

The meat quality measurements included pH, water holding capacity (WHC), and texture profile analysis. All the measurements were done following previously-established methods[76]. The bone strength was measured by testing the energy at maximum load, extension at maximum load, maximum slope and maximum load in a 3-point test using the method described in previous studies[87-88]. Severities of woody breast and white stripling of the breast were scored by five individuals independently on a scale of 1-5 with 1 being a normal breast and 5 being a severely diseased breast. Photos of chicks were taken at the end of week 4 to 6. Feather coverage was scored based on the photos on a scale of 1-5 with 1 being almost no feathering or less than 25% of the body covered, 2 being 25%-50% feather coverage, 3 being 50%-75% feather coverage, 4 being 75%-90% feather coverage and 5 being 100% feather coverage.

2.3.8 Statistical analysis

Software R (version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria) was used for the data analysis. Pen was considered as the experimental unit. Two-way ANOVA was used to evaluate the main effects (the stocking density and concentration of supplemental methionine), and Duncan's multiple range test was used to compare the treatment means. Data were presented as means ± SEM, and the significance level for differences was $P < 0.05$.

Table 1. Composition (g/kg) of experimental diets for broilers

Ingredients, g/kg	Starter	Grower		Finisher	
DL-Met Supplementation	**100%**	**100%**	**130%**	**100%**	**130%**
Corn[§]	558	626	626	664.	664.
Soybean meal	351	291	291	255	255
Soybean oil	37.0	35.5	35.5	40.0	40.0
Dicalcium phosphate	17.8	15.7	15.7	12.7	12.7
Limestone	15.7	13.2	13.2	11.1	11.1
Vit. /min. premix[‡]	5.00	5.00	5.00	5.00	5.00
L-Lysine	4.50	3.60	3.60	3.2.0	3.2.0
DL-Met, >99%[†]	3.60	2.90	3.77	2.60	3.38
Salt	3.10	3.10	3.10	3.50	3.50
L-Valine	1.30	0.90	0.90	0.60	0.60
L-Threonine	1.30	1.00	1.00	0.80	0.80
Choline chloride 60%	1.10	1.10	1.10	1.30	1.30
Sodium bicarbonate	0.80	0.90	0.90	0.00	0.00
L-Isoleucine	0.60	0.40	0.40	0.40	0.40
Analytical values					
ME, kcal/kg	2927	2977	2987	3033	3082
Crude protein %	22.0	19.9	19.5	17.8	17.4
Methionine %	0.65	0.54	0.73	0.49	0.56
Cysteine %	0.35	0.32	0.31	0.29	0.27
Methionine + cysteine %	1.00	0.86	1.04	0.78	0.83
Lysine %	1.47	1.23	1.24	1.09	1.01
Phosphorus %	0.68	0.64	0.61	0.60	0.59
Calcium %[#]	1.05	0.90	0.90	0.76	0.76

[§]Analytical nutrient values of corn: ME, 3320 kcal/kg; crude protein, 77.5 g/kg; lysine, 2.51 g/kg; methionine, 1.64 g/kg. Analytical nutrient values of soybean mean: ME, 2370 kcal/kg; crude protein, 474 g/kg; lysine, 29.3 g/kg; methionine, 66.3 g/kg.

[‡]Vitamin and mineral mixture provided the following nutrients per kilogram of diets: vitamin A, 4,550 IU; vitamin E, 7.5 IU; vitamin D_3, 450 IU; vitamin K, 0.752 mg; riboflavin, 3.75 mg; pantothenic acid, 3 mg; niacin, 15.2 mg; vitamin B_{12} , 0.006 mg; biotin, 0.152 mg; folic acid, 0.376 mg; thiamine, 1.07 mg; pyridoxine, 3.78 mg; choline, 1575 mg; Cu, 12 mg; I, 0.053 mg; Mn, 30.2 mg; Se, 0.09 mg; Zn, 53.0 mg; Fe, 67.8 mg.

[†]DL-methionine (MetAMINO® Evonik Industries, Essen, Germany) with >99% purity

[#]The calcium levels were calculated by the calcium requirement of broilers

Table 2. List of primers used for Quantitative RT-PCR

Gene[§]	Forward (5' to 3') Reverse (5' to 3')
IL6	TGTGCAAGAAGTTCACCGTG A CTCGACGTTCTGCTTTTCG
IL10	GCTGAGGGTGAAGTTTGAGG ATGCTGTGCTGATGACTGGT
TNFα	GTATGTGCAGCAACCCGTAG T GGGCATTGCAATTTGGACA
HSP70	GCAGAGGATGAAGCCAACAG G GTCAAGCGAACTGATCACC
HSP90	ACCAATGGAGGAGGAAGTGG C AATGGTCAGAGTGCGATCG
P38 MAPK	ATAGTTCCCACCCACAACCA C CTTCCCACTGACCACTCAT
AKT1	ATGAAATGATGTGTGGCCGG GCCAAACAATGCCAGCAAAG
JNK	GAGGACGAAGAGGAGGAGGG AAGAGGAGGAGGAGGAGGAG

[§]Abbreviation: IL-6, interlukin-6; IL-10, interlukin-10; TNFα, tumor necrosis factor alpha; HSP70, heat shock protein 70; HSP90, heat shock protein 90; P38 MAPK, P38 mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase.

2.4 Results

2.4.1 Growth performance and plasma health indicators

There were no interaction effects of the stocking density and dietary methionine on any of the growth performance variables measured at any time point. The body weight of birds was not affected by the stocking density in the starter phase (**Table 3**), but the body weight of both the growers and finishers in high density groups were lower ($P < 0.05$) than that in normal density groups (**Figure 3)**. Average daily gain of the birds was not affected by the stocking density in any of the three individual phases; however, the accumulated average daily gain was lowered ($P<0.05$) by the high stocking density. Feed intake was decreased ($P<0.01$) by the high stocking density in all three phases. The birds in the high density groups had lower ($P<0.05$) feed conversion ratio than those in the normal density groups in starter and finisher phases (**Figure 4)**. The concentrations of DL-MET supplementation did not affect the growth performance except the 130% DL-MET supplementation decreased ($P < 0.05$) feed intake of the starters and finishers compared to the 100% DL-MET supplementation.

The high density did not affect the plasma health indicators except for elevating ($P < 0.05$) the concentrations of TG of the growers (**Table 4**). The 130% DL-MET supplementation elevated ($P < 0.01$) plasma AKP levels and TC concentrations of the growers, and the plasma concentrations of uric acid and TG of the finisher broilers compared with the 100% DL-MET supplementation. Plasma concentrations of glucose and TG of the growers were decreased ($P < 0.05$) by the 130% DL-MET

supplementation compared with the 100% DL-MET supplementation. An interaction effect ($P<0.05$) of the stocking density and DL-MET concentration was observed in the grower phase on plasma AKP.

Table 3. Effect of two stocking densities and concentrations of DL- methionine supplementation on growth performance of broilers[§]

Density		Normal		High			P value		
Methionine	Period	100%	130%	100%	130%	SEM	Density	Conc	Interaction
Body Weight, (g/chick)	Starter	345^{a}	336^{ab}	332^{b}	336^{ab} :	2.14	0.13	0.52	0.12
Average Daily Gain, (g/chick/day)	Starter	27.6^{a}	26.8^{ab}	26.8^{ab}	26.5^{b}	0.36	0.13	0.53	0.13
	Grower	57.2^{a}	56.8^{ab}	54.7^{b}	56.3^{ab}	0.82	0.06	0.46	0.20
	Finisher	103	101	98.8	98.5	1.95	0.89	0.55	0.68
Feed Intake, (g/chick/day)	Starter	43.8^{a}	41.8^{b}	37.5^{c}	36.6^{c}	0.62	<0.01	0.03	0.35
	Grower	107^{a}	105^{ab}	100^{c}	101^{bc}	1.63	<0.01	0.84	0.33
Feed/gain	Starter	1.59^{a}	1.56^{a}	1.42^{b}	1.38^{a}	0.02	<0.01	0.12	0.53
	Grower	1.88	1.86	1.83	1.80	0.02	0.06	0.33	0.83
	Total	1.76	1.72	1.76	1.71	0.02	0.77	0.70	0.78

[§]Data are expressed as means (n=10). The main effects (the stocking density and concentration of supple mental DL-methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P<0.05$).

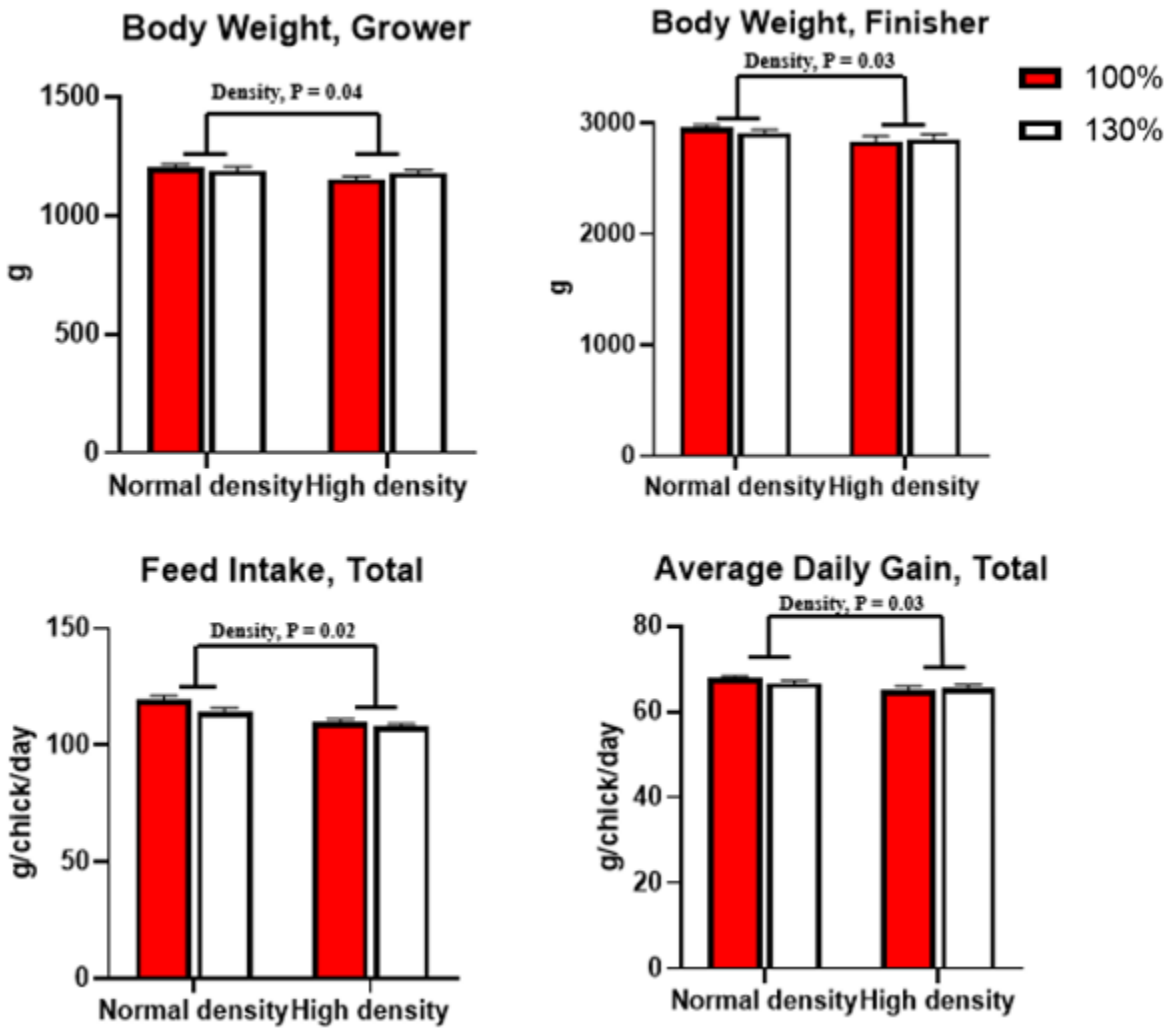

Figure 3. Effects of stocking density on the body weight of the grower and finisher broilers and the accumulative feed intake and average daily gain. Values are means ± SEs, n = 10. A total of 560 Cornish Cross cockerels were divided into four groups: two stocking densities, normal and high density (1.0 and 1.3 bird/ft^2) and two concentrations of supplemental DL-MET, 100 and 130% required methionine. Experimental diets were fed to the birds from the grower phase and the birds were raised for 6 weeks. Two-way ANOVA was used to evaluate the main effects (the stocking density and concentration of supplemental methionine).

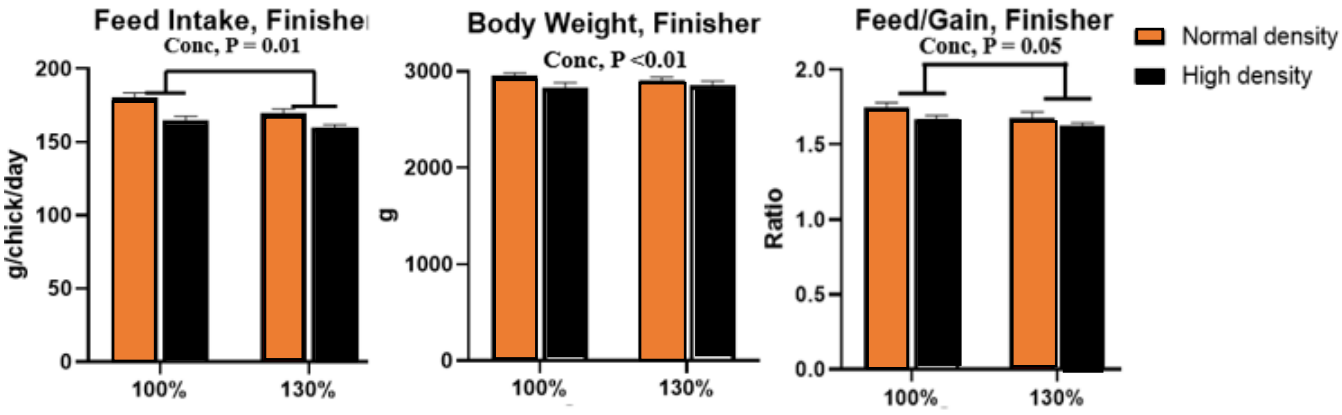

Figure 4. Effects of concentrations of DL-MET supplementation on feed intake, body weight, and feed/gain of the finisher broilers. Values are means ± SEs, n = 10. A total of 560 Cornish Cross cockerels were divided into four groups: two stocking densities, normal and high density (1.0 and 1.3 bird/ft^2) and two concentrations of supplemental DL-MET, 100 and 130% required methionine. Experimental diets were fed to the birds from the grower phase and the birds were raised for 6 weeks. Two-way ANOVA was used to evaluate the main effects (the stocking density and concentration of supplemental methionine).

Table 4. Effect of two stocking densities and concentrations of DL- methionine supplementation on plasma indicators of broilers[§]

Density	Normal		High			P value		
Methionine	100%	130%	100%	130%	SEM	Density	Conc	Interaction
ALT[‡], U/L								
Grower	0.82	0.76	0.75	0.72	0.08	0.55	0.60	0.88
Finisher	0.92	1.04	0.86	1.03	0.11	0.78	0.24	0.82
AKP, U/mL								
Grower	284[a]	287[a]	251[a]	455[b]	31.8	0.05	<0.01	<0.01
Finisher	324	370	314	328	23.0	0.29	0.22	0.51
PIP, mg/dL								
Grower	68.3	70.6	69.2	67.3	1.24	0.34	0.87	0.09
Finisher	55.8	56.6	55.9	53.9	2.12	0.54	0.78	0.54
Glucose, g/L								
Grower	2.94[ab]	2.81[b]	3.11[a]	2.73[b]	0.08	0.57	<0.01	0.14
Finisher	2.55	2.75	2.68	2.66	0.12	0.88	0.49	0.36
Uric acid, mmol/L								
Grower	281	291	288	289	15.8	0.89	0.75	0.79
Finisher	238[b]	287[a]	267[ab]	298[a]	13.1	0.17	<0.01	0.51
TC, mg/dL								
Grower	78.9[b]	89.4[a]	86.1[ab]	90.6[a]	2.46	0.12	<0.01	0.25
Finisher	75.6	71.8	74.4	74.7	2.14	0.69	0.42	0.33
TG, mg/dL								
Grower	40.9[a]	31.7[b]	44.4[a]	40.6[a]	2.29	0.02	0.01	0.28
Finisher	35.0[ab]	40.0[a]	32.3[b]	37.7[ab]	2.00	0.23	0.02	0.93
NEFA, μmol/L								
Grower	0.15	0.15	0.17	0.16	0.01	0.18	0.74	0.69
Finisher	0.13	0.13	0.12	0.11	<0.01	0.09	0.64	0.29

[§]Data are expressed as means (n=10). The main effects (the stocking density and concentration of supplemental DL-methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ s ignificantly ($P < 0.05$).

[‡]ALT, alanine amino transferase; AKP, alkaline phosphatase; PIP, inorganic phosphorus; TC, total cholesterol; TG, triglycerides; NEFA, non-esterified fatty acids.

2.4.2 Lipid and fatty acid profiles

The lipid profiles in the breast, thigh, and liver were neither affected by the stocking density nor the concentration of DL-MET supplementation **(Table 5**). The high density decreased ($P<0.05$) the concentrations of monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), and total fatty acids in the breast and the concentration of MUFA in the adipose tissue of the finisher birds **(Table 6**). The 130% DL-MET supplementation elevated ($P<0.05$) the concentrations of total fatty acids and MUFA in the liver of the finisher birds, while decreased ($P<0.05$) the concentration of MUFA in the breast of the growers (**Figure 5**). There was an interaction effect ($P<0.05$) of the stocking density and DL-MET concentration on the concentration of PUFA in the breast of the grower birds. The breast PUFA concentrations of 130% DL-MET supplementation groups were higher under normal density while lower under high density than those of 100% DL-MET supplementation groups.

Table 5. Effect of two stocking densities and concentrations of DL- methionine supplementation on tissue lipid profile of broilers[§]

Density	Normal		High			P value		
Methionine	100%	130%	100%	130%	SEM	Density	Conc	Interaction
Grower								
Breast								
TC[‡], mg/g protein	2.80	2.83	2.88	2.91	0.05	0.63	0.84	1.00
TG, mg/g protein	16.3	15.2	11.7	11.2	2.58	0.07	0.64	1.00
NEFA, umol/g protein	4.91	5.81	4.85	4.63	0.52	0.09	0.37	0.13
Thigh								
TC, mg/g protein	4.23	4.05	3.97	3.80	0.06	0.29	0.48	0.98
TG, mg/g protein	27.4	24.6	23.6	24.54	1.53	0.62	0.82	0.65
NEFA, umol/g protein	10.1	10.5	8.69	9.02	0.79	0.28	0.78	0.98
Liver								
TC, mg/g protein	12.8	13.2	12.3	14.3	0.74	0.72	0.19	0.40
TG, mg/g protein	25.9	30.1	24.5	28.6	2.28	0.50	0.05	1.00
NEFA, umol/g protein	43.5	44.8	41.7	42.7	1.30	0.60	0.75	0.96
Finisher								
Breast								
TC, mg/g protein	4.83	4.39	4.99	4.81	0.25	0.21	0.17	0.55
TG, mg/g protein	25.0	18.1	17.9	24.9	3.85	0.92	0.90	0.09
NEFA, umol/g protein	4.78^{ab}	3.68^{b}	4.75^{ab}	5.67^{a}	0.71	0.07	0.85	0.05
Thigh								
TC, mg/g protein	3.75	3.82	3.81	3.69	0.06	0.82	0.88	0.54
TG, mg/g protein	19.9	26.6	24.9	29.4	3.52	0.34	0.18	0.80
NEFA, umol/g protein	3.92	4.29	4.93	5.29	0.57	0.08	0.52	0.99
Liver								
TC, mg/g protein	12.8	13.5	14.5	13.6	0.68	0.24	0.95	0.29
TG, mg/g protein	57.2	70.7	77.2	72.0	8.24	0.19	0.63	0.25
NEFA, umol/g protein	32.6	38.3	37.8	43.7	4.28	0.09	0.07	0.98

[§]Data are expressed as means (n=10). The main effects (the stocking density and concentration of supple mental DL-methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used t o compare thetreatment means. Means in the same row without a common letter differ significantly ($P< 0.05$).

[‡]TC, total cholesterol; TG, triglycerides; NEFA, non-esterified fatty acids.

Table 6. Effect of two stocking densities and concentrations of DL- methionine supplementation on tissue fatty acid concentrations of broilers[§]

Density	Normal		High			P value		
Methionine	100%	130%	100%	130%	SEM	Density	Conc	Interaction
Fatty Acid, mg/g tissue								
Liver								
Total								
Grower	15.0	15.5	14.9	16.4	2.05	0.66	0.85	0.82
SFA[‡]								
Grower	7.39	6.85	7.28	7.88	0.95	0.64	0.95	0.59
Finisher	13.5	13.8	12.2	14.4	0.90	0.69	0.18	0.33
MUFA								
Grower	4.59	5.16	4.79	4.85	0.80	0.94	0.73	0.77
PUFA								
Grower	2.99	2.88	3.38	3.67	0.40	0.21	0.86	0.62
Finisher	7.16	8.58	8.12	8.01	0.82	0.43	0.36	0.36
Breast								
Total								
Grower	7.58	7.61[b]	8.23	7.00	0.36	0.95	0.08	0.07
Finisher	9.41a	9.59a	8.74ab	7.40b	0.48	0.01	0.24	0.13
SFA								
Grower	2.41	2.42	2.57	2.26	0.11	1.00	0.19	0.16
Finisher	2.94[a]	2.95[a]	2.70[ab]	2.40[b]	0.13	0.08	0.28	0.26
MUFA								
Grower	2.74[ab]	2.66[ab]	2.95[a]	2.47[b]	0.12	0.95	0.01	0.07
PUFA								
Grower	2.30	2.48	2.61	2.20	0.14	0.91	0.35	0.03
Thigh								
Total								
Grower	9.12	11.3	11.6	11.5	1.10	0.25	0.38	0.34
Finisher	8.34	8.68	8.72	8.73	0.38	0.60	0.67	0.69
SFA								
Grower	2.84	3.36	3.66	3.43	0.39	0.29	0.72	0.37
Finisher	2.67	2.72	2.80	2.65	0.12	0.79	0.67	0.45
MUFA								
Grower	3.19	3.99	4.16	4.21	0.38	0.15	0.29	0.35
Finisher	2.85	2.82	2.88	3.02	0.15	0.46	0.73	0.62

PUFA								
Grower	2.88	3.63	3.52	3.61	0.34	0.38	0.24	0.35
Finisher	2.66	2.95	2.86	2.88	0.15	0.67	0.31	0.36
Adipose tissue								
Total								
Grower	92.3	93.2	91.0	81.6	6.07	0.32	0.51	0.42
Finisher	73.4[a]	71.0[ab]	67.7[ab]	51.6[b]	6.29	0.07	0.17	0.30
SFA								
Grower	26.1	26.7	25.0	22.3	1.91	0.18	0.59	0.41
Finisher	20.0	18.9	19.2	14.3	1.67	1.21	0.09	0.27
MUFA								
Grower	39.0	39.1	38.3	34.5	2.58	0.34	0.49	0.48
PUFA								
Grower	27.1	27.1	27.1	24.3	1.81	0.46	0.47	0.47
Finisher	22.9	23.4	21.8	17.0	2.10	0.09	0.32	0.23

[§]Data are expressed as means (n=10). The main effects (the stocking density and concentration of supplemental DL-methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).

[‡]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

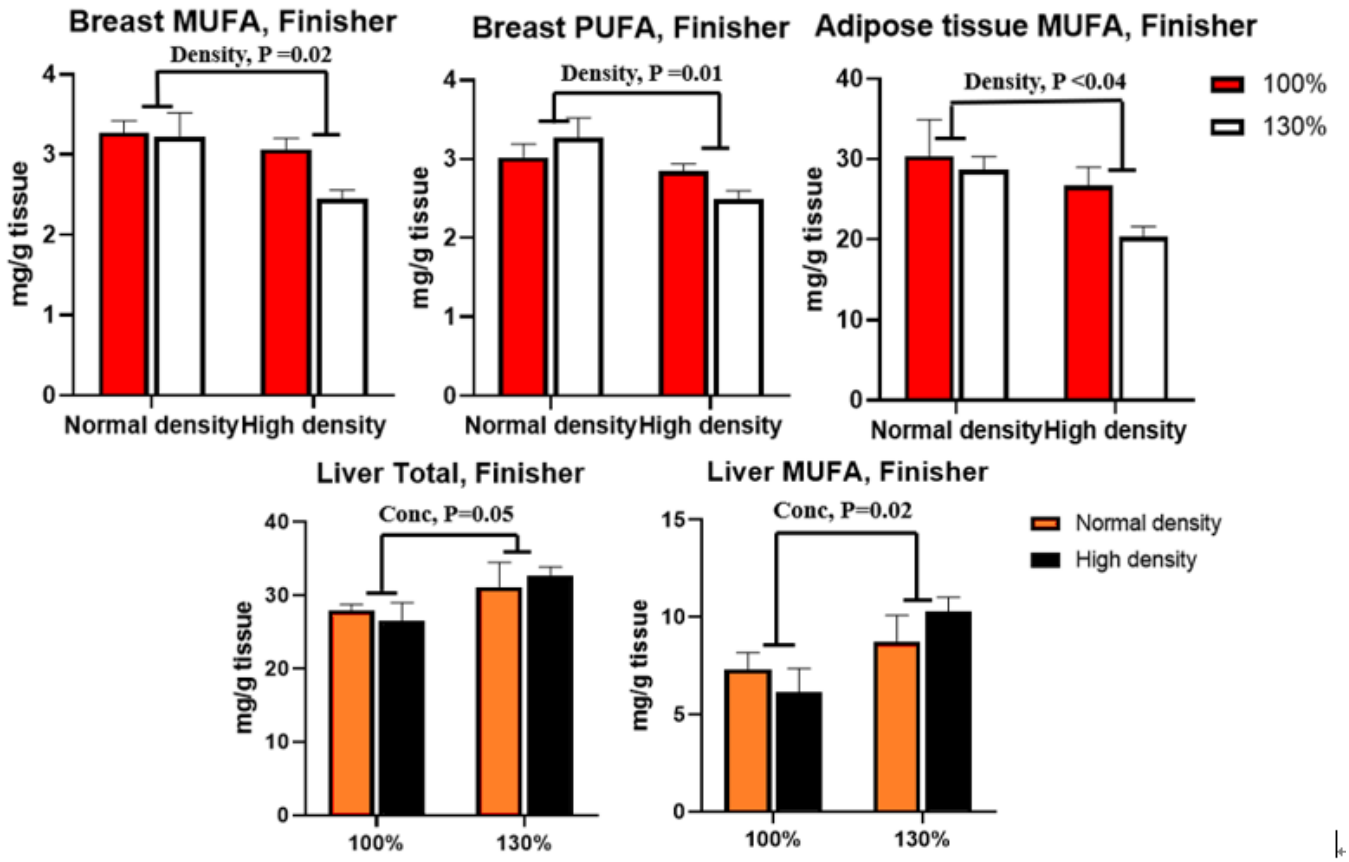

Figure 5. Effects of stocking density (top) and concentrations (bottom) of supplemental DL-MET on concentrations of breast MUFA and PUFA, adipose tissue MUFA, liver MUFA and total fatty acids of the finisher broilers. Values are means ± SEs, n = 10. A total of 560 Cornish Cross cockerels were divided into four groups: two stocking densities, normal and high density (1.0 and 1.3 bird/ft^2) and two concentrations of supplemental DL-MET, 100 and 130% required methionine. Experimental diets were fed to the birds from grower phase and the birds were raised for 6 weeks. Two-way ANOVA was used to evaluate the main effects (the stocking density and concentration of supplemental methionine).

2.4.3 Antioxidant status

The high density decreased ($P<0.05$) the concentration of GSH in the liver but elevated ($P<0.05$) the concentrations of GSH in all other assayed tissues of the growers (**Table 7)**. In the finisher phase, the high density decreased ($P<0.01$) the concentrations of GSSG in the plasma, liver, and breast and the concentration of GSH in the thigh (**Figure 6**). The 130% DL-MET supplementation decreased ($P<0.05$) the concentration of GSSG in the plasma while enhanced both GSH and GSSG in the thigh of the growers compared with the 100% DL-MET supplementation. An interaction effect ($P<0.05$) of the stocking density and DL-MET concentration was found on the concentration of plasma GSH of the finishers. The 130% DL-MET supplementation elevated plasma GSH concentration under normal density while did not affect its concentration under high density. The concentration of MDA in the breast was decreased ($P<0.05$) by the high density. The 130% DL-MET supplementation decreased ($P<0.05$) the concentrations of MDA in the breast and thigh. An interaction effect ($P<0.05$) of the stocking density and DL-MET concentration was found on the concentration of MDA in the breast. The 130% DL-MET supplementation decreased the MDA concentration under normal density while did not affect its concentration under high density.

The high density decreased ($P < 0.05$) the activities of GST in the thigh of both grower and finisher birds **(Table 8)**. The activity of GR in the liver of the growers was decreased ($P < 0.05$) by the high density. The 130% DL-MET supplementation decreased ($P<0.05$) the activity of SOD in the liver of the grower

birds and the activity of GST in the thigh of the finishers while elevated ($P<0.05$) the activity of GR in the thigh of the finisher broilers compared with the 100% DL-MET supplementation. Interaction effects ($P<0.05$) of the stocking density and DL-MET concentration were found on the activities of the GPx and SOD in the thigh and GST in the liver of the growers. The 130% DL-MET supplementation elevated the activity of SOD under high density while did not affect its activity under normal density. The activity of hepatic GST was decreased by the high density in 130% DL-MET supplemental groups while its activity was not affected in 100% DL-MET supplemental groups. There were also interaction effects ($P<0.05$) on the activities of GST in the thigh and adipose tissue of the finisher broilers. The activity of GST in the thigh was decreased by the 130% DL-MET supplementation only under high density and the activity of GST in adipose tissue was increased by the 130% DL-MET supplementation only under normal density.

Table 7. Effect of two stocking densities and concentrations of DL- methionine supplementation on antioxidant status of broilers[§]

Density	Normal		High			P value		
Methionine	100%	130%	100%	130%	SEM	Density	Conc	Interaction
Grower								
Plasma, nmol/ml								
GSSG[‡]	0.42[a]	0.32[b]	0.43[ab]	0.32[b]	1.34	0.71	<0.01	0.74
Liver, µmol/ g								
GSH	0.79[ab]	0.81[a]	0.60[b]	0.70[ab]	0.05	0.02	0.35	0.57
GSSG	0.074	0.059	0.064	0.063	0.013	0.75	0.34	0.36
Breast, µmol/ g								
GSSG	0.030	0.027	0.030	0.033	0.008	0.40	0.99	0.43
Thigh, µmol/ g								
GSSG	0.064[ab]	0.073[a]	0.052[b]	0.068[ab]	0.016	0.15	0.03	0.53
Finisher								
Plasma, nmol/g								
GSSG	0.76[ab]	0.84[a]	0.69[b]	0.66[b]	0.92	<0.01	0.21	0.10
Liver, µmol/ g								
GSH	1.23	1.15	1.14	1.36	0.23	0.46	0.39	0.07
GSSG	0.20[ab]	0.26[a]	0.16[b]	0.18[b]	0.04	<0.01	0.09	0.47
Breast, µmol/ g								
GSH	0.672	0.716	0.648	0.701	0.08	0.64	0.24	0.91
GSSG	0.056[a]	0.051[a]	0.034[b]	0.023[b]	0.011	<0.01	0.15	0.67
Thigh, µmol/ g								
GSH	0.40[ab]	0.50[a]	0.28[b]	0.34[b]	0.08	<0.01	0.06	0.53
GSSG	0.066[ab]	0.077[a]	0.068[ab]	0.063[b]	0.001	0.16	0.48	0.09
MDA, µmol TEP[†] equivalent/mg protein								
Liver	167	157	161	155	12.2	0.81	0.56	0.89
Breast	28.1[a]	9.65[b]	12.5[b]	10.9[b]	2.97	0.03	<0.01	0.02
Thigh	26.5[a]	17.0[b]	21.6[ab]	14.3[b]	2.32	0.14	<0.01	0.66
Adipose tissue	872	687	555	596	132	0.17	0.60	0.43
FRAP, mmol Trolox equivalent/ L plasma								
Plasma	10.1	10.7	10.4	9.1	0.77	0.66	0.45	0.23
Serum amyloid A, ng/ml								
Plasma	90.9	89.2	92.8	85.2	11.6	0.93	0.69	0.80
Corticosterone, ng/ml								
Plasma	364	405	345	396	47.7	0.81	0.38	0.93

Protein carbonyl, nmol/ mg								
Liver	3.88	3.85	3.73	4.42	0.36	0.57	0.34	0.33

[§]Data are expressed as means (n=10). The main effects (the stocking density and concentration of sup plemental DL-methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ signific antly ($P<0.05$).

[‡]GSH, glutathione; GSSG, glutathione disulfide; MDA, malondialdehyde; FRAP, ferric reducing ability of plasma. [†]TEP, 1,1,3,3-tetraethoxypropane.

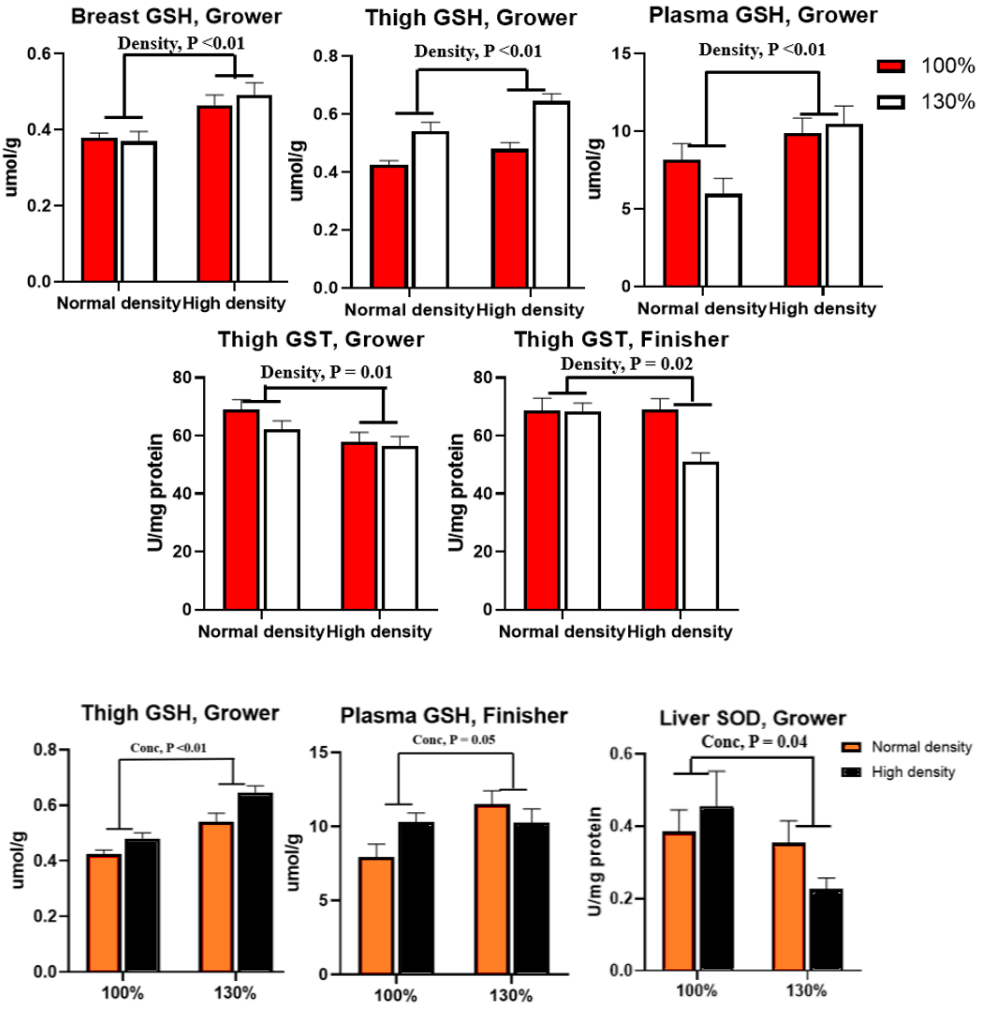

Figure 6. Effects of stocking density (top) and concentrations (bottom) of supplemental DL-MET on the concentrations of breast and thigh GSH of the growers, plasma GSH of the growers and finishers, and activities of liver SOD of the growers and thigh GST of the growers and finishers.

Values are means ± SEs, n = 10. A total of 560 Cornish Cross cockerels were divided into four groups: two stocking densities, normal and high density (1.0 and 1.3 bird/ft^2) and two concentrations of supplemental DL-MET, 100 and 130% required methionine. Experimental diets were fed to the birds from the grower phase and the birds were raised for 6 weeks. Two-way ANOVA was used to evaluate the main effects (the stocking density and concentration of supplemental methionine).

Table 8. Effect of two stocking densities and concentrations of DL- methionine supplement ation on antioxidant enzymes activity of broilers[§]

Density	Normal		High			P value		
Methionine	100%	130%	100%	130%	SEM	Density	Conc	Interaction
Grower								
Breast, U/mg protein								
GR[‡],	3.44	3.53	4.72	4.08	0.25	0.08	0.59	0.47
GPx	18.1	18.2	16.3	16.6	0.58	0.14	0.85	0.92
GST	42.6	49.1	44.6	43.8	1.42	0.57	0.32	0.21
SOD	0.94	0.93	1.00	0.50	0.10	0.36	0.20	0.21
Thigh, U/mg protein								
GR	4.23	3.87	4.44	4.57	0.21	0.31	0.80	0.59
GPx	34.1	28.7	27.7	34.6	1.19	0.92	0.73	0.01
GST	69.1^{a}	62.3^{ab}	58.0^{b}	56.5^{b}	1.70	0.01	0.20	0.41
SOD	0.43^{ab}	0.19^{b}	0.26^{b}	0.62^{a}	0.05	0.12	0.47	<0.01
Liver, U/mg protein								
GR	12.8	11.9	10.7	10.7	0.38	0.04	0.55	0.55
GPx	106	127	110	102	6.53	0.43	0.62	0.30
GST	205^{ab}	223^{a}	211^{ab}	188^{b}	5.04	0.13	0.78	0.04
Adipose tissue, U/mg protein								
GR	20.6	17.3	20.7	20.2	0.65	0.25	0.18	0.28
GPx	176^{b}	189^{ab}	184^{ab}	209^{a}	4.94	0.14	0.05	0.54
GST	101^{b}	158^{a}	162^{a}	148^{ab}	9.25	0.93	0.22	0.06
SOD	2.22	1.91	2.46	1.37	0.27	0.81	0.25	0.52
Finisher								
Breast, U/mg protein								
GR[‡],	4.11	4.23	5.49	4.34	0.28	0.20	0.37	0.27
GPx	20.2	21.6	22.1	19.7	1.02	0.98	0.82	0.37
GST	34.1	40.3	40.4	34.0	1.59	1.00	0.98	0.05
SOD	0.21	0.30	0.23	0.19	0.02	0.33	0.49	0.13
Thigh, U/mg protein								
GR	2.82^{b}	4.18^{a}	4.10^{a}	4.36^{a}	0.21	0.07	0.04	0.16
GPx	13.7	15.4	14.5	16.2	0.72	0.58	0.24	0.99
SOD	0.22	0.17	0.14	0.21	0.02	0.65	0.88	0.26
Liver, U/mg protein								
GR	15.3	15.2	15.6	13.1	0.43	0.29	0.13	0.17
GPx	24.5	25.8	26.6	22.9	0.71	0.78	0.39	0.09
GST	208^{ab}	204^{ab}	218^{a}	190^{b}	4.29	0.81	0.06	0.15

SOD	0.72	0.67	0.91	0.86	0.06	0.09	0.65	0.99
Adipose tissue, U/mg protein								
GR	8.83	10.3	13.0	9.19	0.77	0.53	0.29	0.19
GPx	118	124	127	139	3.95	0.15	0.23	0.75
GST	23.5^{b}	64.4^{a}	40.5^{ab}	29.0^{b}	5.18	0.32	0.12	<0.01
SOD	2.21	2.66	1.34	1.85	0.34	0.26	0.51	0.97

§Data are expressed as means (n=10). The main effects (the stocking density and concentration of supplemental DL-methionine) were analyzed by two-way ANOVA and Duncan's multiple ra nge test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P<0.05$).

‡ GR, glutathione reductase; GPx, glutathione peroxidase; GST, glutathione transferase; SOD, su peroxide dismutase.

2.4.4 Inflammation-related gene expressions

The mRNA levels of the analyzed inflammation-related genes were not affected by neither the density nor the concentration of DL-MET supplementation except the mRNA levels of HSP90 were lower ($P < 0.05$) in 130% DL-MET supplementation groups than those in the 100% DL-MET supplementation groups **(Table 9**).

2.4.5 Meat quality, bone strength, breast muscle myopathy, mortality rate, and feather coverage

The high density decreased ($P<0.05$) the WHC of the breast muscle but increased ($P<0.01$) the springiness of the breast and thigh and hardness of the thigh (**Table 10**). The 130% DL-MET supplementation decreased ($P<0.01$) the WHC of the breast but increased ($P<0.05$) the hardness of the breast and springiness of the thigh. Both the high density and the 130% DL-MET supplementation increased ($P<0.05$) the maximum load of the tibia of the grower chicks (**Table 11)**. The breast myopathy scores, mortality rate, and feather coverage scores were not affected by the high density nor the methionine supplementations (**Table 12, 13)**

Table 9. Effect of two stocking densities and concentrations of DL- methionine supplementation s on liver inflammation-related gene expressions of finisher broilers[§]

Density	Normal		High			P value		
Methionine	100%	130%	100%	130%	SEM	Density	Conc	Interaction
IL-6[‡]	1.00	1.03	0.54	0.53	0.14	0.16	0.96	0.96
IL-10	1.00	1.00	0.69	0.61	0.13	0.31	0.92	0.92
TNFα	1.00	0.71	0.74	0.69	0.07	0.37	0.29	0.45
HSP70	1.00	0.73	0.82	0.69	0.07	0.50	0.22	0.65
HSP90	1.00	0.78	1.00	0.86	0.06	0.67	0.04	0.61
AKT	1.00	0.99	0.99	1.00	0.05	0.99	0.99	0.91
P38MAPK	1.00	1.12	1.66	1.42	0.14	0.14	0.88	0.58
JNK	1.00	1.10	1.22	0.80	0.12	0.92	0.52	0.35

[§]Data are expressed as means (n=10). The main effects (the stocking density and concentration of su pplemental DL-methionine) were analyzed by two-way ANOVA and Duncan's multiple range test wa s used to compare the treatment means. Means in the same row without a common letter differ sign ificantly ($P<0.05$).

[‡]IL-6, interleukin 6; IL-10, interleukin 10; TNFα, tumor necrosis factor alpha, HSP70, heat shock pr otein 70; HSP90, heat shock protein 90; AKT, protein kinase b; P38MAPK, P38 mitogen-activated p rotein kinase; JNK, c-Jun N-terminal kinase.

Table 10. Effect of two stocking densities and concentrations of DL- methionine supplementation on meat quality of finisher broilers[§]

Density	Normal		High			P value		
Methionine	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Dressing Percentage, %	79.9	79.6	79.5	81.5	4.69	0.47	0.43	
Meat to bone ratio, Thigh, %	88.0^{b}	88.7^{a}	87.6^{b}	88.3^{ab}	<0.01	0.21	0.05	0.93
Breast								
pH	6.07	6.01	5.94	6.02	0.04	0.11	0.88	0.07
WHC[‡], %	42.9^{a}	40.9^{a}	40.8^{a}	37.6^{b}	<0.01	<0.01	<0.01	0.57
Springiness	0.81^{ab}	0.79^{b}	0.83^{a}	0.82^{a}	0.01	<0.01	0.15	0.65
Hardness	3252^{b}	4465^{a}	3846^{ab}	3888^{ab}	279	0.96	0.03	0.04
Thigh								
pH	5.87	5.95	5.95	5.93	0.06	0.61	0.68	0.39
WHC	42.1	40.5	41.3	40.3	0.01	0.53	0.10	0.73
Springiness	0.81^{b}	0.84^{a}	0.84^{a}	0.84^{a}	0.01	0.01	0.06	0.05
Hardness	3250^{b}	3836^{b}	4617^{a}	3470^{b}	264	0.03	0.20	<0.01

[§]Data are expressed as means (n=10). The main effects (the stocking density and concentration of supple mental DL-methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used t o compare the treatment means. Means in the same row without a common letter differ significantly (P< 0.05).

[‡]WHC, water holding capacity.

Table 11. Effect of two stocking densities and concentrations of DL- methionine supplementation on tibia strength of broilers[§]

Density	Normal		High			P value		
Methionine	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Grower								
Bone Weight, g	8.48	7.91	9.12	8.73	0.53	0.36	0.17	0.86
Energy at Maximum Load, J	0.28	0.27	0.26	0.28	<0.01	0.87	0.93	0.69
Extension at Maximum Load, mm	2.21	1.96	2.04	2.29	0.16	0.87	0.60	0.11
Maximum Slope, mm/N	0.019	0.015	0.019	0.017	0.002	0.57	0.27	0.67
Maximum Load, N	207^{b}	231^{b}	222^{b}	283^{a}	13.5	0.02	<0.01	0.16
Finisher								
Bone Weight, g	19.3	20.6	19.2	18.7	0.73	0.16	0.56	0.22
Energy at Maximum Load, J	0.35	0.40	0.38	0.31	0.03	0.38	0.73	0.058
Extension at Maximum Load, mm	1.98	1.89	1.99	1.73	0.13	0.59	0.19	0.49
Maximum Slope, mm/N	0.014	0.015	0.012	0.012	0.003	0.38	0.24	0.35
Maximum Load, N	375	331	366	335	30.6	0.94	0.23	0.82

[§]Data are expressed as means (n=10). The main effects (the stocking density and concentration of supple mental DL-methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).

Table 12. Effect of two stocking densities and concentrations of DL- methionine supplementation on breast muscle myopathy scores (1-5), incidence of tibial dyschondroplasia (TD) , and mortality of broilers[§]

Density	Normal		High			P value		
Methionine	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Woody Breast[‡]	1.80	1.85	1.95	2.0	0.70	0.20	0.81	1.00
White Stripling	1.85	1.95	2.3	1.7	0.90	0.64	0.24	0.11
TD, %	8.89	7.78	7.50	6.67	2.34	0.65	0.73	0.96
Mortality, %	1.1	0.7	0.9	0.9	0.50	1.00	0.56	0.56

[§]Data are expressed as means (n=10). The main effects (the stocking density and concentration of supplemental DL-methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).

[‡]Breasts were scored for woody breast and white stripling on a scale of 1-5 with 1 being a normal breast and 5 being a severely diseased breast.

Table 13. Effect of two stocking densities and concentration of DL- methionine supplementations on feather coverage[‡] of broilers[§]

Density	Normal		High			P value		
Methionine	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Week 4	3.49	3.53	3.50	3.60	0.13	0.78	0.60	0.83
Week 5	4.23	4.20	4.28	4.13	0.10	0.90	0.39	0.54
Week 6	4.63	4.68	4.60	4.60	0.081	0.55	0.77	0.77

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).

[‡]Photos of chicks were taken at weeks 5 and 6. Feather coverage was scored based on the photos on a scale of 1-5 with 1 being almost no feathering or less than 25% of the body covered, 2 being 25%-50% feather coverage, 3 being 50%-75% feather coverage, 4 being 75%-90% feather coverage and 5 being 100% feather coverage.

2.5 Discussion

This current study was conducted to fill in the gap of the literature regarding the effects of 30% extra methionine supplementation as DL-MET in the grower and finisher diets of broilers subjected to a high stocking density challenge. In the current study, high stocking density conditions depressed performance parameters of broilers including body weight, feed intake, and average daily gain which was in agreement with previous studies[6, 8]. Although the extra methionine supplementation decreased the feed intake of the birds, the body weight and average daily gain were not affected. These results might indicate that the extra methionine supplementation could improve the feed conversion efficiency of the broilers through reducing oxidative stress.

The major finding of the current study was that the extra supplemental DL-MET could improve the antioxidant status of the broilers. One proof of the improved antioxidant status was the elevated concentrations of GSH in the thigh of the growers and plasma of the finishers in the 130% DL-MET groups. The enhancement in GSH concentrations with extra dietary methionine has also been reported in previous studies[89-90] as methionine can be indirectly utilized to synthesize GSH in the one carbon cycle[22]. The improvement in antioxidant status was also indicated by the decreased MDA concentrations, signifying decreased lipid peroxidation, in the breast and thigh. This effect of decreased MDA

accumulation within broiler tissues has been seen before when adding dietary antioxidants such as vitamin E, selenium, or astaxanthin to broiler diets[75, 91].Surprisingly, the high stocking density elevated the GSH concentrations in plasma, breast, and thigh of the growers and decreased the GSSG concentrations in plasma, liver, and breast of the finishers. These results were in contrast with the previous study that raised broilers under high stocking density although this might be due to differences in the severity of the oxidative stress [74]. Previous researches have also shown that oxidative stress would induce the formation of GSH and enhance the expression of GSH synthetic enzymes [92-93]. The different responses on GSH and GSSG concentrations of broilers exposed to high stocking density might indicate the GSH metabolism of the broilers can change depending on the severity of the stress. It is also interesting to find that although the GSH concentrations were elevated in plasma, breast, and thigh of the growers, its concentration decreased in the liver. Since GSH is synthesized mainly in the liver and then transported into different tissues[94], during oxidative stress it is possible that more GSH is transported out of the liver and mobilized quicker than it can be synthesized. If the liver is mobilizing GSH faster than it can produce it, this could result in a lower GSH concentration in the liver while higher GSH concentrations in the plasma and other tissues.

Conversely, although the high stocking density increased the concentrations of GSH in the tissues, there was a reduction in the activities of GST in the thigh, GR in the liver of the

growers, and GPx in the thigh of the finishers. Previous studies have also reported decreased GPx activity in the blood serum of broilers subjected to high stocking density [73-74]. The decrease in the activities of antioxidant enzymes suggested an increased oxidative stress due to the high stocking density. Intriguingly, the antioxidant enzyme activities in the assayed tissues responded to the supplemental methionine differently. The extra DL-MET supplementation decreased the activity of SOD in the liver of the growers and GST in the thigh of the finishers. Such decrease in activities of antioxidant enzymes was in agreement with previous studies that supplemented broiler diets with astaxanthin or methionine[76-77]. However, the activities of GPx in the adipose tissue of the growers and GR in the thigh of the finishers were elevated by the extra supplemental DL-MET. Future research needs to be performed to explain these variations and understand the mechanism of how broilers under high stocking density utilize the intrinsic antioxidant system and extrinsic antioxidant supplementations to cope with oxidative stress.

In the current study, the extra methionine supplementation decreased the mRNA level of HSP90, a gene which is involved with antioxidant and inflammatory response. During oxidative stress conditions, the heat shock proteins are upregulated [70-71]. The decreased mRNA level of HSP90 in this study could be due to the amelioration of oxidative status brought by the extra methionine supplementation. This was also in agreement to the results found in previous studies that supplemented broilers under high temperature with vitamin

E and selenium [95]. However, the high density did not affect the mRNA levels of other inflammation related genes which contradicts results regarding oxidative stress in dendritic cells and cancer cells reported by previous researchers[96-97]. One possible reason for the largely unchanged mRNA levels of the inflammation related genes was that the birds under density stress were not experiencing a severe enough inflammation to upregulate the expressions of these inflammatory response genes, this was furthered signified by a lack of change in the serum amyloid A and plasma corticosterone with density level. The other reason could be that the changes of gene expressions were transient while only one timepoint were measured in this study, thus we did not observe difference in the mRNA levels.

The decreased concentrations of fatty acids in breast and adipose tissue of the finishers under high stocking density indicated that the broilers were experiencing changes in metabolism which would indicate some degree of oxidative stress. Interestingly, it was demonstrated that extra methionine supplementation exerted protective effects on the fatty acids in the broiler tissues. This was shown by the higher concentrations of total fatty acids and MUFA in the liver. Similar changes in fatty acid compositions were noticed with dietary supplementation of antioxidants in broilers, providing evidence that extra methionine can protect fatty acids from being oxidized [75, 98]. The protective effects were also manifested by the lower MDA concentrations in the breast and thigh. Since fatty acids

especially long chain unsaturated fatty acids are susceptible to oxidation, the enhancement in hepatic fatty acid concentrations provided another proof of the antioxidant potential of extra methionine supplementation [43].

In conclusion, the supplementation of 30% extra DL-MET supplementation in the corn-soybean meal based diets for grower and finisher broilers under high stocking density improved the antioxidant status by increasing GSH levels and decreasing lipid peroxidation in the tissues. Supplemental extra methionine increased the mRNA levels of heat shock protein 90 in the liver and improved the feed conversion ratio of the broilers. These results indicated that the extra supplementation of DL-MET in broiler diets could be beneficial to broiler under high stocking density.

CHAPTER THREE

Supplemental methionine in broiler raised at high ambient temperature[2]

3.1 Abstract

This study was to explore metabolic effects of two forms and concentrations of supplemental methionine in grower and finisher diets for broiler chickens raised at high temperature. Cornish Cross cockerels (total = 360, day-old) were divided into four groups (10 pens/treatment, 9 birds/pen) and fed with 100 or 130% required methionine in the diets as DL-methionine (DL-MET) or 2-hydroxy-4-(methylthio)butanoate (HMTBA). The room was maintained at 4 to 13oC above the suggested thermoneutral temperature. The higher concentration of both DL-MET and HMTBA enhanced ($P < 0.05$) hepatic GSH concentrations of the growers and plasma ferric reducing ability of the finishers. The DL-MET-fed growers had greater ($P < 0.05\%$) muscle GSH and hepatic unsaturated fatty acid concentrations than those fed HMTBA. Expression of inflammation-related genes in the liver of finishers was affected ($P < 0.05$) by interaction effects of the methionine form and concentration. In conclusion, effects of the extra methionine

[2] Guanchen Liu, Andrew D. Magnuson, Tao Sun, Samar A. Tolba, Charles Starkey, Rose Whelan and Xin Gen Lei, Supplemental methionine exerted chemical form-dependent effects on antioxidant status, inflammation-related gene expression, and fatty acid profiles of broiler chicks raised at high ambient temperature, Journal of Animal Science, 2019, https://doi.org/10.1093/jas/skz348.

supplementation on the high ambient temperature-related metabolic responses of broilers varied with their age and(or) tissue and the methionine form.

3.2 Introduction

In the US, the major broiler production is in the southern States with high ambient temperature during the summer[9]. Thus, fast-growing broilers raised in those states are likely to experience heat stress and suffer from impaired growth performance, compromised immune responses, and poor health status[10-11]. High ambient temperature will induce excessive ROS production in the broilers and therefore results in oxidative stress, and the impaired performance and wellbeing of the broilers may be the consequences of oxidative stress [46, 99]. Antioxidants such as vitamin E, carotenoids, and sulfur-containing amino acids (methionine and cysteine) are known for scavenging reactive oxygen species (ROS) generated from oxidative stress[17-18].

Methionine is an essential sulfur-containing amino acid required for tissue growth and protein synthesis and is the first-limiting amino acid to broiler chicks fed commercial corn and soybean meal diets. Synthetic DL-MET and HMTBA are two commonly-used methionine supplements in animal diets. Chemically, HMTBA is the hydroxyl analog of methionine (the amino group in methionine is replaced with a hydroxyl group). To be utilized in the body, , HMTBA needs to be converted into L-methionine in a two-step

process that takes place mainly in the liver and to some extent in other tissues including small intestine and kidney[28-29]. It has been a long-time debate if MTBA is less bioavailable than DL-MET due to differences in absorption, conversion and utilization[30-32]. Notably, methionine has been shown to protect the brain[100-101], liver[102-103], and muscle[104-105] from oxidative damages. This is because methionine exposed on the surface of proteins is readily oxidized into methionine sulfoxide, which protects the integrity and function of other critical residues in the proteins from oxidation[19-21]. Moreover, methionine can be converted into homocysteine in the one-carbon cycle in the liver[22]. Thereafter, homocysteine can be converted into cysteine through transsulfuration[22]. Cysteine is not only a potent antioxidant itself, but also a precursor for the synthesis of glutathione (GSH)[23-25], a potent scavenger of ROS[26-27]. Dietary supplemental DL-MET and HMTBA have been shown to improve anti-oxidant status, anti-inflammatory response, growth performance, and wellbeing of broiler chicks[30, 33-35]. However, few studies have determined if those benefits could be enhanced by elevating the methionine supplementation and(or) vary with its chemical form in broilers exposed to a high ambient temperature.

Therefore, this study was conducted to test a working hypothesis that elevating supplemental DL-MET and HMTBA from the 100% to the 130% of the required digestible methionine concentrations into corn-soybean meal-based grower and finisher

diets for broilers would help the animals cope with the high ambient temperature-induced metabolic stress. Our objectives were to compare if the two forms and concentrations of supplemental methionine exerted similar or different effects on: 1) growth performance, meat quality, feather coverage, and bone strength of broilers; and 2) antioxidant status, health indicators, inflammation-related gene expression, and lipid and fatty acid profiles in several tissues of broilers.

3.3 Materials and methods

3.3.1 Animals, diets, and management

All protocols in this study were approved by the Cornell University Institutional Animal Care and Use Committee. A total of 360 (day-old) Cornish Cross cockerels were purchased from Moyer's Chicks (Quakertown, PA). The birds were reared in 1 m^2 floor-pens in environmentally-controlled rooms with 2:22 h dark-light cycles during the entire experiment. The temperature in the room for the starter period was set at optimal according to the industrial guide[106]. Thereafter, the temperature was kept at 31°C (in comparison with the suggested steady decreases from 27 to 18ºC over the age of day 14 – 42) to impose heat stress on the birds. The birds were randomly allotted into four groups (10 pens/treatment, 9 birds/pen) based on the initial body weights. All birds were fed the same corn and soybean meal based starter diet (day 0 – 10). During the grower (day 11 - 22) and finisher (day 23 – 42) periods, different experimental diets were fed to the birds.

Supplemental DL-MET was > 99% pure (MetAMINO®, Evonik Industries, Essen, Germany). A liquid methionine hydroxyl analogue product containing 88% DL-HMTBA was diluted at 2:1 with Sipernat silica (a food grade ingredient consists 99% of silicon dioxide provided by Evonik SIPERNAT®) and created a dry product for feed mixing with a final product concentration of 58% HMTBA. To meet the recommended (100%) methionine+cysteine requirement by broilers based on AMINOChick® 2.0 (Evonik Nutrition & Care GmbH, Germany), 3.09 and 2.77g of DL-MET/kg diet was supplemented to the grower and finisher diets, respectively. For the HMTBA treatment groups, the concentration of HMTBA in the product (58%) as well as the equimolar bioefficacy of 75% for HMTBA compared with DL-MET[31] was considered so that 7.19 and 6.44 g of HMTBA product/kg diet was supplemented to the grower and finisher diets, respectively. To prepare diets with 130% additional supplemental methionine, 4.02 g MET/kg or 9.34 g HMTBA/kg was added into the grower diets and 3.60 g MET/kg or 8.37 g HMTBA/kg was added into the finisher diets. Actual concentrations of all nutrients except for Ca in all experimental diets were determined by analysis (Evonik Nutrition & Care GmbH) and the analyzed values are shown in **Table 14**. Animals were given free access to water and feed throughout the experiment.

3.3.2 Sample collection

Body weights and feed intakes of each pen were recorded weekly and at the end of each phase. At the end of grower and finisher periods, two chickens from each pen were euthanized by carbon dioxide followed by cervical dislocation. Blood was collected from the heart by heparinized needles. Plasma was then separated by centrifugation at 12000g for 15 min at 4°C (Beckman GS-6R centrifuge, Brea, CA). After the liver, breast, thigh, and abdominal adipose tissue were collected, a portion of the tissues was frozen in liquid nitrogen and then stored at -80°C for gene expression measurement. The remaining tissues were kept on dry ice and later stored at -20°C for meat quality test and biochemical analyses. After removing the muscle, tendon, and ligament, the left tibia from each bird was collected and stored at -20°C for strength test.

3.3.3 Plasma health indicators

Plasma activities of alanine aminotransferase (ALT), alkaline phosphatase (AKP), and tartrate-resistant acid phosphatase (TRAP) and concentrations of plasma inorganic phosphorus (PIP), glucose, total cholesterol (TC), triglyceride (TG), non-esterified fatty acid (NEFA), and uric acid were analyzed following methods described in previous studies[76-77].

3.3.4 Tissue lipid and fatty acid profiles

The lipid profiles (TC, TG, and NEFA) and fatty acid profiles were measured in the liver, adipose tissue, breast, and thigh following methods in previous studies[76-77]. The gas chromatography-mass spectrometry (model HP 5890 A with an HP 5970 series mass-selective ion-monitoring detector, Hewlett-Packard, Palo Alto, CA) with the internal standard of tritridecanoin was used to analyze the fatty acid profiles.

3.3.5 Tissue and plasma antioxidant status

Concentrations of total GSH, glutathione disulfide (GSSG) in the plasma, liver, breast and thigh and concentrations of malondialdehyde (MDA) in the livers, adipose, breast and thigh were assayed using methods of previous studies[76-77]. The ferric reducing ability of plasma (FRAP) was determined using Benzie and Strain's method[79]. Concentrations of protein carbonyl in the liver were determined using a method developed by Oliver et al[80]. An ELISA kit (Cayman Chemical, Ann Arbor, MI) was used to determine concentrations of corticosterone in plasma. Activities of glutathione peroxidase (GPx), glutathione transferase (GST), glutathione reductase (GR), and superoxide dismutase (SOD) were measured in the breast, thigh, liver and adipose tissue using methods adapted from previous studies[81-84].

3.3.6 Quantitative real-time PCR

Abundances of interlukin-6 (IL-6), interlukin-10 (IL-10), tumor necrosis factor alpha (TNFα), heat shock protein 70 (HSP70), heat shock protein 90 (HSP90), protein kinase B (AKT), P38 mitogen-activated protein kinases (P38MAPK), and c-Jun N-terminal kinase (JNK) mRNA in the liver were determined. Primers used for these tested genes are listed in **Table 2.** Total mRNA was isolated and purified from the liver using TRIzol Reagent (Life Technologies, Carlsbad, CA) following the established method[85]. The mRNA reverse transcription was done by the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). The Real-time qPCR (7900 HT; Applied Biosystems) and the $2^{-\text{delta delta Ct } (\Delta\Delta Ct)}$ equation[86] were then used to quantify the mRNA expression levels.

3.3.7 Meat quality, bone strength, breast muscle myopathy, and feather coverage

The meat quality of breast and thigh muscle including pH, water holding capacity, and texture profile was assessed following previously-established methods[76]. The bone strength was measured by testing the energy at maximum load, extension at maximum load, maximum slope and maximum load in a 3-point test using the method described in previous studies[87-88]. Before collecting the breast tissues, severities of woody breast and while stripling of the breast were scored by five individuals independently on a scale of 1-5 with 1 being a normal breast and 5 being a severely diseased breast. Photos of birds

were taken at weeks 5 and 6. Feather coverage was scored based on the photos on a scale of 1-5 with 1 being almost no feathering or less than 25% of the body covered, 2 being 25%-50% feather coverage, 3 being 50%-75% feather coverage, 4 being 75%-90% feather coverage and 5 being 100% feather coverage.

3.3.8 Statistical analyses

Software R (version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria) was used for the data analysis. Pen was considered as the experimental unit. Two-way ANOVA was used to evaluate the main effects (the chemical form and concentration of supplemental methionine), and Duncan's multiple range test was used to compare the treatment means. Data were presented as means ± SEM, and the significance level for differences was $P < 0.05$

Table 14. Composition (g/kg) of experimental diets for broilers

Guanchen Liu

Ingredients, g/kg	Starter	Grower				Finisher			
	DL-MET	DL-MET		HMTBA		DL-MET		HMTBA	
	100%	100%	130%	100%	130%	100%	130%	100%	130%
Corn[§]	557.6	627	626	623	621	665	664	661	659
Soybean meal	357	297	297	297	297	261	261	261	261
Soybean oil	30.6	30.0	30.0	30.0	30.0	35.5	35.5	35.5	35.5
Dicalcium phosphate	20.7	18.5	18.5	18.5	18.5	14.9	14.9	14.9	14.9
Limestone	15.4	10.5	10.5	10.5	10.5	9.0	9.0	9.0	9.0
Vit. /min. premix[‡]	2.90	2.90	2.90	2.90	2.90	2.90	2.90	2.90	2.90
L-Lysine	4.25	3.42	3.42	3.42	3.42	3.00	3.00	3.00	3.00
DL-Met, >99%[†]	3.84	3.09	4.02	0.00	0.00	2.77	3.60	0.00	0.00
DL-HMTBA, 58%	0.00	0.00	0.00	7.19	9.34	0.00	0.00	6.44	8.37
Salt	3.11	3.10	3.10	3.10	3.10	3.48	3.48	3.48	3.48
L-Valine	1.07	0.66	0.66	0.66	0.66	0.35	0.35	0.35	0.35
L-Threonine	1.26	0.95	0.95	0.95	0.95	0.84	0.84	0.84	0.84
Choline chloride 60%	1.07	1.11	1.11	1.11	1.11	1.27	1.27	1.27	1.27
Sodium bicarbonate	0.82	0.89	0.89	0.89	0.89	0.00	0.00	0.00	0.00
L-Isoleucine	0.46	0.25	0.25	0.25	0.25	0.26	0.26	0.26	0.26
Analytical values									
ME, kcal/kg	2947	2943	2950	2950	2935	3047	3024	3011	3005
Crude protein %	21.0	20.0	20.0	20.0	20.0	19.0	18.3	18.2	18.8
Methionine %	0.66	0.57	0.64	0.36	0.32	0.53	0.60	0.31	0.30
HMTBA %	0.00	0.00	0.00	0.41	0.58	0.00	0.00	0.39	0.54
Cysteine %	0.32	0.31	0.31	0.31	0.31	0.30	0.29	0.29	0.29
Methionine+cysteine %	0.98	0.88	0.95	0.67	0.63	0.83	0.89	0.60	0.59
Lysine %	1.35	1.27	1.22	1.22	1.26	1.20	1.10	1.13	1.15
Phosphorus %	0.62	0.61	0.62	0.61	0.57	0.57	0.56	0.55	0.55
Calcium %[#]	1.05	0.90	0.90	0.90	0.90	0.76	0.76	0.76	0.76

[§]Analytical nutrient values of corn: ME, 3320 kcal/kg; crude protein, 77.5 g/kg; lysine, 2.51 g/kg; methionine, 1.64 g/kg. Analytical nutrient values of soybean mean: ME, 2370 kcal/kg; crude protein, 474 g/kg; lysine, 29.3 g/kg; methionine, 66.3 g/kg.

[‡]Vitamin and mineral mixture provided the following nutrients per kilogram of diets: vitamin A, 4,550 IU; vitamin E, 7.5 IU; vitamin D 3 , 450 IU; vitamin K, 0.752 mg; riboflavin, 3.75 mg; pantothenic acid, 3 mg; niacin, 15.2 mg; vitamin B_{12} , 0.006 mg; biotin, 0.152 mg; folic acid, 0.376 mg; thiamine, 1.07 mg; pyridoxine, 3.78 mg; choline, 1575 mg; Cu, 12 mg; I, 0.053 mg; Mn, 30.2 mg; Se, 0.09 mg; Zn, 53.0 mg; Fe, 67.8 mg.

[†]DL-methionine (MetAMINO® Evonik Industries, Essen, Germany) with >99% purity and a liquid methionine hydroxy analogue product containing 88% DL-HMTBA, diluted at 2:1 with sipernat silica to create a dry product for feed mixing and resulting in a final product concentration of 58% HMTBA, were used as the sources of supplemental methionine in the diets.

[#]Calcium levels were calculated based on the calcium requirement for broilers

3.4 Results

3.4.1 Growth performance and plasma health indicators

Neither the form nor the concentration of supplemental methionine affected the body weight, average daily gain or feed conversion ratio throughout the study (**Table 15**). The feed intake of the broilers fed DL-MET was 7% higher ($P < 0.05$) than that of the broilers fed HMTBA during finisher phase. The 130% methionine supplementation decreased ($P < 0.05$) plasma uric acid concentration of the finisher birds and PIP concentrations of both growers and finisher birds, but elevated ($P < 0.01$) plasma AKP activity of the finishers compared with the 100% methionine supplementation (**Table 16**.). In the finisher phase, the plasma AKP activity and uric acid concentration of the DL-MET-fed groups were 25% and 14% higher ($P < 0.05$), respectively, than those of HMTBA-fed groups. The plasma ALT activity of the finishers was 32% lower ($P < 0.05$) in the DL-MET-fed groups than that in HMTBA-fed groups. In the grower phase, an interaction effect ($P < 0.05$) of methionine form and concentration was found on the plasma glucose concentration.

Table 15. Effect of two chemical forms and concentrations of methionine supplementations on growth performance of broilers[§]

Form		DL-MET		HMTBA			P value		
Concentration	Period	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Body weight,	Starter	324	331	332	329	3.82	0.44	0.47	0.49
(g/chick)	Grower	1378	1410	1421	1407	21.3	0.35	0.68	0.29
	Finisher	2612	2631	2593	2559	53.1	0.40	0.89	0.63
					:				
Average daily gain,	Starter	26.0	26.7	26.7	26.5	0.35	0.43	0.47	0.19
(g/chick/day)	Grower	62.0	63.5	64.1	63.4	1.16	0.39	0.76	0.38
	Finisher	82.3	81.4	80.0	76.8	2.40	0.17	0.42	0.65
	Total	59.4	61.5	62.0	62.0	1.12	0.19	0.37	0.37
	Total(G-F)	73.9	74.2	73.0	71.9	1.70	0.37	0.85	0.70
Feed intake,	Starter	39.3	39.9	39.7	40.1	0.49	0.62	0.34	0.86
(g/chick/day)	Grower	107	107	107	106	1.36	0.90	0.80	0.49
	Finisher	142^{ab}	144^{a}	134^{b}	134^{ab}	2.82	<0.01	0.43	0.97
	Total	102	103	99.1	99.6	1.13	0.02	0.51	0.79
	Total(G-F)	123	124	120	120	1.53	0.02	0.59	0.76
Feed/gain	Starter	1.52	1.48	1.49	1.53	0.02	0.70	0.88	0.15
	Grower	1.72	1.69	1.68	1.67	0.02	0.15	0.49	0.57
	Finisher	1.73	1.78	1.72	1.78	0.05	0.93	0.34	0.98
	Total	1.73	1.74	1.71	1.74	0.03	0.60	0.55	0.69
	Total(G-F)	1.73	1.78	1.72	1.78	0.05	0.62	0.53	0.85

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly (P<0.05).

Table 16. Effect of two chemical forms and concentrations of methionine supplementations on plasma indicators of broilers[§]

Form	DL-MET		HMTBA			P value		
Concentration	100%	130%	100%	130%	SEM	Form	Conc	Interaction
ALT[‡], U/L								
Grower	0.97	0.86	1.08	0.97	0.07	0.16	0.14	0.99
Finisher	1.38	1.27	1.74	1.76	0.16	0.03	0.79	0.73
AKP, U/mL								
Grower	726	769	720	775	49.1	0.99	0.34	0.90
Finisher	390^{bc}	654^{a}	343^{c}	493^{b}	59.2	<0.01	<0.01	0.15
PIP, mg/dL								
Grower	65.1	62.9	66.0	61.2	2.11	0.77	0.02	0.38
Finisher	59.9^{a}	54.6^{b}	56.4^{ab}	55.1^{ab}	1.55	0.39	0.05	0.22
Glucose, g/L								
Grower	4.23	4.68	4.72	4.40	0.12	0.43	0.51	0.03
Finisher	3.42	3.30	3.38	3.61	0.13	0.51	0.31	0.05
Uric acid, mmol/L								
Grower	342	339	329	346	13.1	0.88	0.47	0.84
Finisher	565	449	450	435	27.0	0.04	0.02	0.08
TC, mg/dL								
Grower	76.7	72.8	73.3	73.6	2.12	0.55	0.41	0.33
Finisher	96.7	103	103	107	4.56	0.35	0.42	0.96
TG, mg/dL								
Grower	40.8	46.7	43.6	51.8	3.97	0.35	0.08	0.75
Finisher	37.7^{a}	29.0^{b}	33.7^{ab}	33.6^{ab}	2.01	0.65	0.06	0.05
NEFA, μmol/L								
Grower	0.10	0.10	0.11	0.09	<0.01	0.10	0.19	0.59
Finisher	0.11	0.11	0.11	0.12	<0.01	0.32	0.91	0.78

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly (P<0.05).

[‡]ALT, alanine amino transferase; AKP, alkaline phosphatase; PIP, inorganic phosphorus; TC, total cholesterol; TG, triglycerides; NEFA, non-esterified fatty acids.

3.4.2 Lipid and fatty acid profiles

Neither the form nor the concentration of methionine supplementation affected the tissue TC, TG, or NEFA concentrations (**Table 17**) except for that the TG concentration in the thigh was higher ($P < 0.05$) in the HMTBA-fed groups than that in the DL-MET-fed groups. Compared with the 100% methionine supplementation, the 130% methionine supplementation elevated ($P < 0.01$) concentrations of MUFA in the liver of the growers but decreased ($P < 0.05$) concentrations of MUFA, and PUFA in the adipose tissue of the finishers (**Table 18**). Concentrations of total fatty acid, SFA, MUFA, and PUFA in the liver of the DL-MET-fed birds were higher ($P < 0.05$) than those of the HMTBA-fed birds in the grower phase (**Figure 7**). In the finisher phase, concentrations of all measured fatty acids in the breast and the SFA concentration in the thigh of the DL-MET-fed birds were lower ($P < 0.05$) than those in the breast and thigh of the HMTBA-fed birds. Interaction effects ($P < 0.05$) of the methionine form and concentration were found on concentrations of MUFA and PUFA in the adipose tissue of the growers.

Table 17. Effect of two chemical forms and concentrations of methionine supplementations on tissue lipid profiles of finisher broilers[§]

Form	DL-MET		HMTBA			P value		
Concentration	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Breast								
TC[‡], mg/g protein	4.13	3.79	3.70	4.20	0.26	0.95	0.73	0.13
TG, mg/g protein	19.8	22.0	24.5	23.4	2.54	0.24	0.83	0.51
NEFA, μmol/g protein	7.83	7.24	7.02	7.19	0.61	0.54	0.76	0.58
Thigh								
TC, mg/g protein	2.28	2.06	2.43	2.21	0.15	0.31	0.15	0.99
TG, mg/g protein	17.1^{b}	23.0^{ab}	28.4^{ab}	31.1^{a}	4.16	0.02	0.31	0.70
NEFA, μmol/g protein	3.15	4.15	4.85	3.78	0.55	0.28	0.90	0.08
Liver								
TC, mg/g protein	9.92	9.32	9.15	8.33	0.57	0.13	0.22	0.85
TG, mg/g protein	21.5	19.2	20.0	17.6	1.74	0.40	0.21	0.95
NEFA, μmol/g protein	48.0	46.9	44.8	47.4	2.18	0.54	0.73	0.39
Adipose tissue								
TC, mg/g protein	55.1	62.9	69.5	65.8	9.42	0.39	0.86	0.57
TG, mg/g protein	1125	1284	1078	1175	164	0.64	0.44	0.85
NEFA, μmol/g protein	159	197	209	188	30.2	0.49	0.80	0.34

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P<0.05$).

[‡]TC, total cholesterol; TG, triglycerides; NEFA, non-esterified fatty acids.

Table 18. Effect of two chemical forms and concentrations of methionine supplementations on fatty acid concentrations of broilers[§]

Form	DL-MET		HMTBA			P value		
Concentration	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Fatty acids, mg/g tissue								
Liver								
Total								
Finisher	14.9	15.8	15.1	15.1	1.57	0.92	0.80	0.80
SFA[‡]								
Grower	8.62	8.72	6.66	7.41	0.77	0.05	0.58	0.67
Finisher	7.26	8.06	7.70	7.76	0.61	0.90	0.55	0.58
MUFA								
Finisher	4.21	4.12	3.30	4.17	0.67	0.57	0.61	0.53
PUFA								
Finisher	3.04	3.13	3.15	3.18	0.17	0.69	0.77	0.91
Breast								
Total								
Grower	7.71	8.16	7.24	6.84	0.62	0.18	0.97	0.51
Finisher	5.72[b]	5.71[b]	7.93[a]	8.01[a]	0.40	<0.01	0.93	0.91
SFA								
Grower	2.30	2.66	2.35	2.42	0.16	0.55	0.23	0.39
Finisher	2.02[b]	2.00[b]	2.61[a]	2.62[a]	0.13	<0.01	0.94	0.90
MUFA								
Grower	2.62	3.03	3.09	2.63	0.23	0.95	0.78	0.08
PUFA								
Grower	2.32	2.56	2.29	2.05	0.19	0.17	0.98	0.23
Thigh								
Total								
Grower	7.80	7.79	7.98	8.23	0.42	0.47	0.76	0.75
Finisher	8.63	7.90	8.85	8.32	0.45	0.49	0.19	0.83
SFA								
Grower	2.32	2.50	2.56	2.56	0.14	0.35	0.55	0.57
Finisher	2.79[ab]	2.46[b]	3.08[a]	2.85[ab]	0.14	0.02	0.04	0.71
MUFA								
Grower	2.87	2.75	3.24	3.01	0.14	0.08	0.31	0.73
Finisher	3.06	2.71	3.38	2.96	0.15	0.10	0.06	0.83
PUFA								

Grower	2.45	2.37	2.55	2.70	0.13	0.15	0.77	0.42
Finisher	2.95	2.70	3.06	2.79	0.16	0.55	0.13	0.95
Adipose tissue								
Total								
Grower	64.9	78.6	73.1	66.3	7.31	0.57	0.99	0.39
Finisher	86.3	79.0	88.5	83.1	4.52	0.49	0.19	0.83
SFA								
Grower	16.5	21.9	18.7	17.2	2.00	0.35	0.80	0.28
Finisher	27.9	26.3	29.6	28.5	1.52	0.21	0.39	0.86
MUFA								
Grower	23.5	29.8	30.3	22.7	2.59	0.93	0.67	0.01
PUFA								
Grower	18.9	22.8	22.4	17.7	1.88	0.63	0.62	0.05

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P<0.05$).

[‡]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

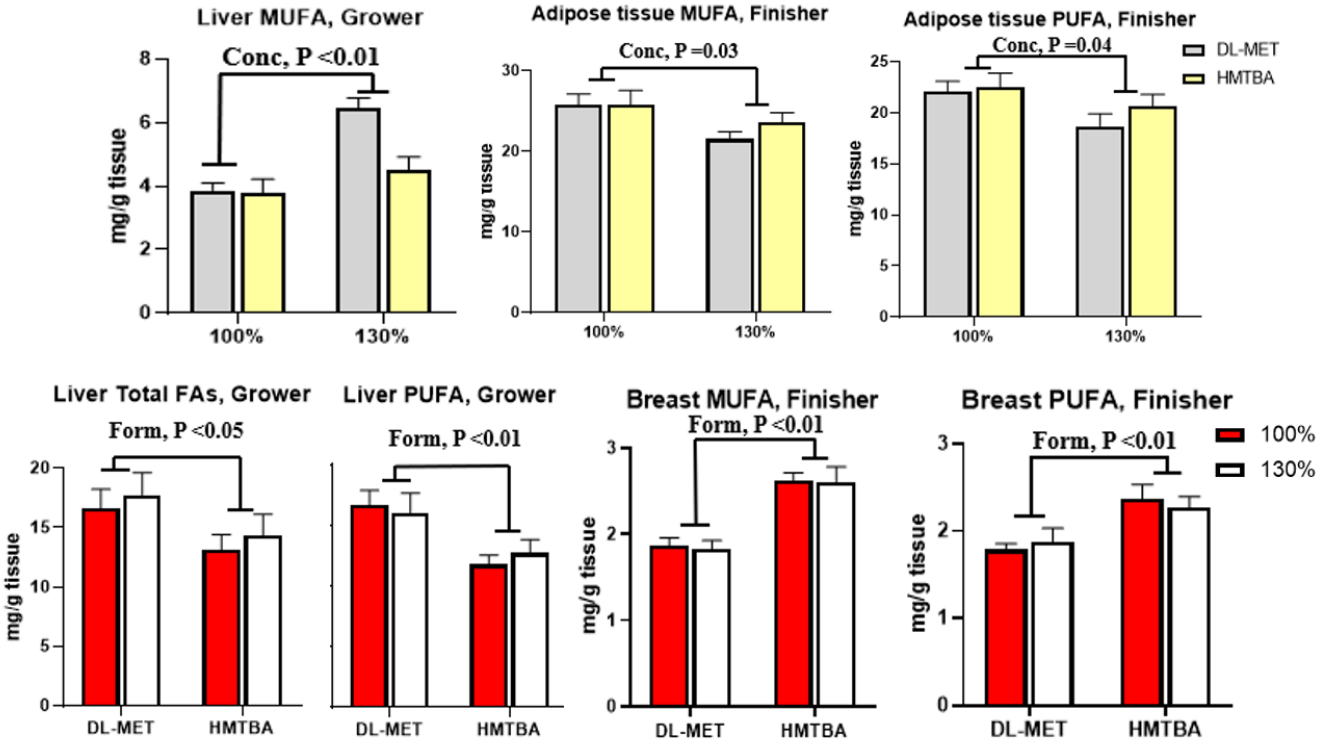

Figure 7. Effect of concentrations (top) and chemical forms (bottom) of methionine supplementations on concentrations of liver MUFA, total fatty acids, and PUFA of the growers, adipose tissue MUFA and PUFA and breast MUFA and PUFA of the finishers. Values are means ± SEs, n = 10. A total of 360 Cornish Cross cockerels were divided into four groups: two chemical forms of supplemental methionine, DL-MET or HMTBA and two concentrations, 100 or 130% of required methionine. From the grower phase, the birds were fed experimental diets and raised in high ambient temperature at 31°C. The birds were raised for 6 weeks. Two-way ANOVA was used to evaluate the main effects (the chemical form and concentration of supplemental methionine).

3.4.3 Antioxidant status

In the growers, the 130% methionine supplementations elevated ($P < 0.05$) GSH concentrations in the liver and GSSG concentrations in the liver and thigh compared with the 100% methionine supplementations (**Table 19**). In the finishers, the 130% methionine supplementations decreased ($P < 0.01$) concentrations of GSH in the plasma and GSSG in the liver, but elevated ($P < 0.05$) concentrations of GSSG in breast compared with the 100% methionine supplementations (**Figure 8**). The GSH concentration in the breast and GSH and GSSG concentrations in the thigh of the DL-MET-fed growers were higher ($P < 0.05$) than those of the HMTBA-fed birds. The 130% methionine supplementations enhanced ($P < 0.05$) FRAP compared with the 100% methionine supplementations in the finishers. Interaction effects ($P < 0.05$) of the methionine form and concentration were found on the hepatic GSSG concentration of the growers and GSSG concentrations in the breast and thigh of the finishers.

The 130% methionine supplementation decreased ($P < 0.05$) activities of GPx in the thigh and GST and SOD in the adipose tissue of the growers compared with the 100% methionine supplementation (**Table 20)**. Similarly, the 130% methionine supplementations decreased ($P < 0.05$) activities of GPx in the liver and GPx and GST in the adipose tissue of the finishers, compared with the 100% methionine supplementations

(**Figure 9**). Activities of GPx in the breast, GR in the thigh, GR and GST in the adipose tissue, and all assayed antioxidant enzymes in the liver of growers were lower ($P < 0.05$) in the DL-MET-fed birds than those in the HMTBA-fed birds. But the GPx activity in the thigh of growers fed DL-MET was higher ($P < 0.05$) than that of the HMTBA-fed birds. Interaction effects ($P<0.05$) of the methionine form and concentration were found on activities of SOD in the breast, GST in the liver, and GR in the adipose and thigh of the growers and GPx in the breast of the finishers.

Table 19. Effect of two chemical forms and concentrations of methionine supplementations on antioxidant status of broilers[§]

Form	DL-MET		HMTBA			P value		
Concentration	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Grower								
Plasma, nmol/ml								
GSH[‡]	3.72	4.45	3.52	3.67	1.69	0.82	0.71	0.64
GSSG	0.31	0.42	0.35	0.37	0.02	0.94	0.32	0.52
Liver, µmol/g								
GSSG	3.17^{ab}	4.31^{a}	3.08^{b}	2.08^{b}	0.38	0.71	<0.01	<0.01
Breast, µmol/g								
GSSG	0.016^{ab}	0.019^{a}	0.010^{b}	0.017^{ab}	0.002	0.15	0.06	0.52
Thigh, µmol/g								
GSSG	0.053^{b}	0.097^{a}	0.039^{b}	0.062^{ab}	0.010	0.02	<0.01	0.29
Finisher								
Plasma, nmol/g								
GSH	2.88^{a}	0.86^{b}	2.37^{a}	1.90^{ab}	0.41	0.67	0.01	0.09
GSSG	0.25^{ab}	0.30^{a}	0.32^{a}	0.24^{b}	0.05	0.67	0.05	0.25
Liver, µmol/g								
GSH	2.37^{b}	2.86^{ab}	3.24^{ab}	3.57^{a}	0.37	0.24	0.36	0.91
GSSG	0.11^{a}	0.083^{ab}	0.069^{b}	0.051^{b}	0.007	0.05	<0.01	0.61
Breast, µmol/g								
GSH	0.195	0.23	0.20	0.24	0.04	0.99	0.28	0.76
GSSG	0.032^{b}	0.078^{a}	0.047^{ab}	0.053^{ab}	0.010	0.86	0.02	0.04
Thigh, µmol/g								
GSH	0.25	0.36	0.21	0.26	0.04	0.06	0.07	0.43
GSSG	0.059^{a}	0.036^{b}	0.028^{b}	0.047^{ab}	0.005	0.16	0.67	<0.01
MDA, µmol TEP[†] equivalent/mg protein								
Liver	29.1	26.6	27.3	29.2	2.54	0.30	0.87	0.11
Breast	23.5	23.1	25.4	22.6	2.58	0.91	0.92	0.43
Thigh	66.7	74.4	80.2	71.2	5.02	0.82	0.57	0.64
Adipose tissue	1202	1400	1364	1107	152	0.67	0.85	0.14
FRAP, mmol Trolox equivalent/L plasma								
Grower	16.7	17.6	16.5	15.6	1.07	0.31	0.99	0.42
Serum corticosterone, ng/ml								
Finisher	179	198	219	258	47.7	0.32	0.56	0.85

Liver protein carbonyl, nmol/ mg								
Finisher	1.67	1.81	1.84	1.49	0.36	0.88	0.82	0.48

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).

[‡]GSH, glutathione; GSSG, glutathione disulfide; MDA, malondialdehyde; FRAP, ferric reducing ability of plasma.

[†]TEP, 1,1,3,3-tetraethoxypropane.

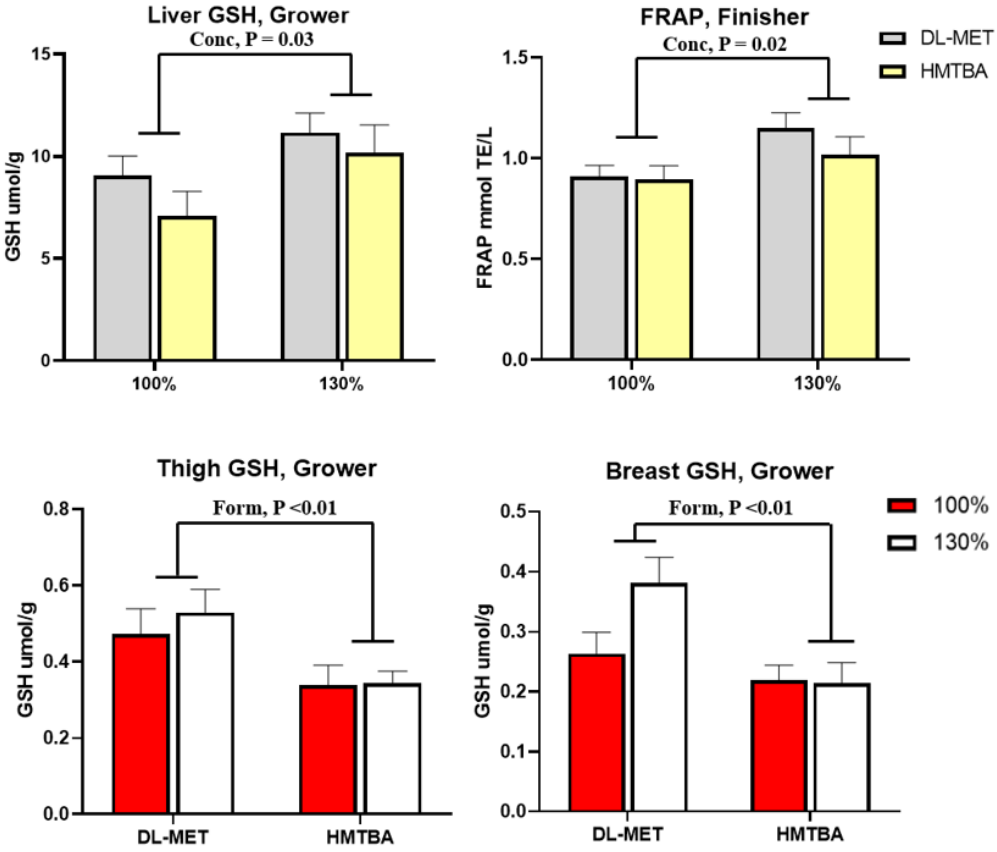

Figure 8. Effect of concentrations (top) and chemical forms (bottom) of methionine supplementations on concentrations of liver, breast, and thigh GSH of the growers and FRAP levels of the finisher. Values are means ± SEs, n = 10. A total of 360 Cornish Cross cockerels were divided into four groups: two chemical forms of supplemental methionine, DL-MET or HMTBA and two concentrations, 100 or 130% of required methionine. From the grower phase, the birds were fed experimental diets and raised in high ambient temperature at 31°C. The birds were raised for 6 weeks. Two-way ANOVA was used to evaluate the main effects (the chemical form and concentration of supplemental methionine).

Table 20. Effect of two chemical forms and concentrations of methionine supplementations on antioxidant enzymes activities of broilers[§]

Form	DL-MET		HMTBA			P value		
Concentration	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Grower								
Breast, U/mg protein								
GR[‡]	4.07	4.34	3.46	3.69	0.31	0.34	0.70	0.97
GST	45.3	43.5	44.0	40.7	2.07	0.64	0.56	0.87
SOD	1.46^{a}	0.99^{ab}	0.96^{b}	1.14^{ab}	0.06	0.30	0.40	0.05
Thigh, U/mg protein								
GR	89.8^{b}	141^{a}	146^{a}	140^{a}	6.26	0.01	0.05	0.01
GPx	12.1^{a}	9.90^{b}	8.17^{bc}	6.50^{c}	0.46	<0.01	<0.01	0.70
GST	80.9	83.8	80.7	73.5	1.94	0.18	0.57	0.20
SOD	0.21	0.19	0.32	0.22	0.03	0.29	0.20	0.28
Liver, U/mg protein								
GR	11.5^{ab}	9.91^{b}	13.9^{a}	13.0^{ab}	0.58	0.02	0.26	0.76
GST	136^{c}	123^{c}	177^{b}	219^{a}	8.73	<0.01	0.29	0.04
SOD	0.043^{b}	0.038^{b}	0.067^{ab}	0.091^{a}	0.013	0.02	0.52	0.34
Adipose tissue, U/mg protein								
GR	10.8^{b}	4.62^{b}	12.0^{b}	31.4^{a}	1.92	<0.01	0.05	<0.01
GPx	41.7	35.2	41.4	49.7	3.63	0.35	0.91	0.32
GST	57.2^{b}	69.0^{b}	64.9^{b}	92.4^{a}	3.89	0.03	<0.01	0.25
SOD	7.21^{ab}	6.29^{b}	9.46^{a}	5.78^{b}	0.50	0.35	0.02	0.14
Finisher								
Breast, U/mg protein								
GR[‡]	5.69	9.14	7.32	6.84	0.64	0.79	0.25	0.13
GPx	27.1	42.6	42.4	31.9	3.13	0.71	0.68	0.04
GST	79.3	106	92.2	97.7	5.48	0.83	0.16	0.35
SOD	1.05	1.50	1.04	1.37	0.14	0.81	0.18	0.83
Thigh, U/mg protein								
GR	14.0	12.1	11.9	11.8	0.46	0.31	0.22	0.33
GPx	15.5	16.3	14.6	14.4	0.48	0.14	0.77	0.60
GST	79.8	73.9	81.7	77.0	1.75	0.48	0.14	0.87
SOD	0.33	0.21	0.31	0.39	0.03	0.22	0.73	0.16
Liver, U/mg protein								
GR	14.9	15.8	16.2	15.6	0.37	0.47	0.82	0.34
GST	241	220	237	229	5.85	0.82	0.24	0.61
SOD	0.19	0.18	0.10	0.20	0.02	0.33	0.21	0.12

Adipose tissue, U/mg protein								
GR	21.4^{ab}	19.5^{b}	23.0^{ab}	29.3^{a}	1.01	0.52	0.53	0.12
SOD	34.0^{b}	35.3^{ab}	47.2^{a}	44.3^{ab}	2.27	0.02	0.86	0.62

§Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).

‡ GR, glutathione reductase; GPx, glutathione peroxidase; GST, glutathione transferase; SOD, superoxide dismutase.

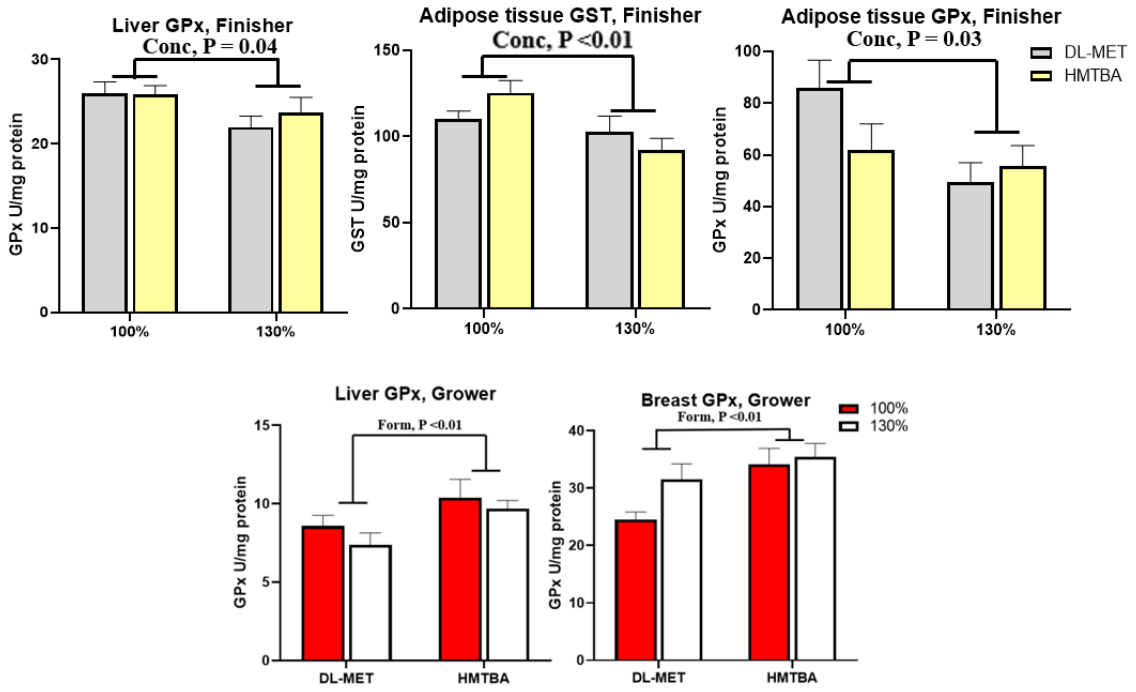

Figure 9. Effect of concentrations (top) and chemical forms (bottom) of methionine supplementations on activities of liver GPx of the growers and finishers, breast GPx of the growers, and adipose tissue GST and GPx of the finishers. Values are means ± SEs, n = 10. A total of 360 Cornish Cross cockerels were divided into four groups: two chemical forms of supplemental methionine, DL-MET or HMTBA and two concentrations, 100 or 130% of required methionine. From the grower phase, the birds were fed experimental diets and raised in high ambient temperature at 31°C. The birds were raised for 6 weeks. Two-way ANOVA was used to evaluate the main effects (the chemical form and concentration of supplemental methionine).

3.4.4 Inflammation-related gene expressions

The concentration and form of methionine supplementations exerted an interaction effect ($P < 0.05$) on the mRNA levels of all tested genes in the liver except for TNFα and JNK (**Table 21**). The 130% DL-MET supplementation increased mRNA levels of IL-6, AKT and P38MAPK compared with the 100% DL-MET supplementation, but the concentrations of HMTBA supplementation did not affect their mRNA levels. Conversely, mRNA levels of IL-10 and HSP70 were lowered in the 130% HMTBA supplemented birds than those of the 100% HMTBA supplemented birds, but their levels were unaffected by the concentrations of DL-MET supplementation. The mRNA level of HSP90 was higher in the DL-MET birds than that of the HMTBA-fed ones at the 130% supplementation, while no difference on its mRNA level between chemical forms was observed at the 100% supplementation. The mRNA levels of JNK and HSP90 were lower in DL-MET fed birds than those of the HMTBA fed birds.

3.4.5 Meat quality, breast muscle myopathy, bone strength and feather coverage

The dressing percentage, meat to bone ratio, pH and WHC of both breast and thigh muscles were unaffected by either the form or concentration of supplemental methionine (**Table 22**). The 130% methionine supplementations enhanced ($P < 0.05$) the chewiness of the breast compared with the 100% methionine supplementations. Neither the form nor the concentration of supplemental methionine affected the breast muscle myopathy scores

(**Table 23**). The 130% methionine supplementations decreased ($P < 0.05$) the energy at maximum load and the extension at maximum load of the tibia of the finishers compared with the 100% methionine supplementations (**Table 24**). The energy at maximum load of tibia of the growers was higher ($P < 0.01$) in the DL-MET-fed groups than that in HMTBA-fed groups. The feather coverage scores of the broilers at weeks 5 and 6 were not affected by either the form or the concentration of methionine supplementations (**Table 25**).

Table 21. Effect of two chemical forms and concentrations of methionine supplementations on liver inflammation-related gene expressions of finisher broilers[§]

Form	DL-MET		HMTBA			P value		
Concentration	100%	130%	100%	130%	SEM	Form	Conc	Interaction
IL-6[‡]	1.00^{b}	2.52^{a}	2.41^{a}	1.42^{ab}	0.38	0.39	0.76	<0.01
IL-10	1.00^{ab}	1.13^{ab}	1.67^{a}	0.45^{b}	0.19	0.48	<0.01	<0.01
TNFα	1.00	1.17	1.23	1.09	0.13	0.53	0.94	0.23
HSP70	1.00^{ab}	1.78^{a}	1.60^{a}	0.68^{b}	0.23	0.09	0.26	<0.01
HSP90	1.00^{ab}	1.48^{a}	1.10^{ab}	0.79^{b}	0.18	0.02	0.37	0.02
AKT	1.00^{b}	1.77^{a}	1.46^{ab}	1.10^{ab}	0.22	0.32	0.14	0.01
P38MAPK	1.00^{b}	1.32^{a}	1.11^{ab}	0.92^{b}	0.12	0.16	0.51	0.01
JNK	1.00^{b}	1.16^{ab}	1.42^{a}	1.36^{ab}	0.12	0.03	0.70	0.40

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).
[‡]IL-6, interleukin 6; IL-10, interleukin 10; TNFα, tumor necrosis factor alpha, HSP70, heat shock protein 70; HSP90, heat shock protein 90; AKT, protein kinase b; P38MAPK, P38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase.

Table 22. Effect of two chemical forms and concentrations of methionine supplementations on meat quality of finisher broilers[§]

Form	DL-MET		HMTBA			P value		
Concentration	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Dressing percentage, %	78.8	79.0	79.7	79.0	<0.01	0.41	0.44	0.26
Meat to bone ratio of thigh, %	87.7	88.8	87.5	87.6	<0.01	0.15	0.24	0.31
Breast								
Weight, g	588	608	596	584	12.8	0.77	0.87	0.53
pH	6.17	6.21	6.22	6.15	0.06	0.87	0.83	0.29
WHC[‡], %	0.42	0.41	0.39	0.41	0.02	0.33	0.82	0.45
Springiness	0.77	0.75	0.77	0.76	0.01	0.90	0.19	0.69
Hardness	5544[a]	5507[a]	3940[b]	5263[a]	388	0.01	0.08	0.08
Chewiness	2388[ab]	2545[a]	1938[b]	2542[a]	183	0.21	0.04	0.24
Thigh								
Weight, g	557	569	546	531	18.0	0.10	0.69	0.65
pH	6.11	6.12	6.09	6.03	0.07	0.25	0.63	0.56
WHC, %	0.61	0.62	0.63	0.60	0.01	0.70	0.71	0.09
Springiness	0.87	0.86	0.88	0.88	0.02	0.32	0.54	0.68
Hardness	5551	5568	4317	5521	457	0.18	0.23	0.23
Chewiness	3424	3312	3245	3688	361	0.80	0.66	0.44

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplem ental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to co mpare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).

[‡]WHC, water holding capacity.

Table 23. Effect of two chemical forms and concentrations of methionine supplementations on breast muscle myopathy scores of broilers[§]

Form	DL-MET		HMTBA			P value		
Concentration	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Woody Breast[‡]	1.2	1.7	1.4	1.7	0.21	0.20	0.81	1.00
White Stripling	1.4	1.7	1.7	1.5	0.17	0.64	0.24	0.11

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).

[‡]Breasts were scored for woody breast and white stripling on a scale of 1-5 with 1 being a normal breast and 5 being a severely diseased breast.

Table 24. Effect of two chemical forms and concentrations of methionine supplementations on tibia strength of broilers[§]

Form	DL-MET		HMTBA			P value		
Concentration	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Grower								
Bone weight, g	11.6	11.6	11.6	11.5	0.29	0.90	0.73	0.90
Energy at maximum load, J	0.33[ab]	0.40[a]	0.29[b]	0.30[b]	0.03	0.01	0.14	0.23
Extension at maximum load, mm	2.90	3.13	3.11	2.87	0.10	0.95	0.70	0.02
Maximum slope, mm/N	0.10	0.10	0.099	0.091	0.010	0.97	0.31	0.30
Maximum load, N	306	338	289	287	13.5	0.08	0.44	0.38
Finisher								
Bone weight, g	19.5	18.8	19.7	18.9	0.57	0.87	0.21	0.98
Energy at maximum load, J	0.26	0.20	0.29	0.20	0.03	0.61	0.01	0.65
Extension at maximum load, mm	2.72	2.43	2.75	2.42	0.15	0.94	0.04	0.91
Maximum slope, mm/N	0.086	0.077	0.077	0.074	0.010	0.39	0.35	0.61
Maximum load, N	285	272	356	297	21.7	0.02	0.30	0.74

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).

Table 25. Effect of two chemical forms and concentrations of methionine supplementations on feather coverage[‡] of broilers[§]

Form	DL-MET		HMTBA			P value		
Concentration	100%	130%	100%	130%	SEM	Form	Conc	Interaction
Week 5	4.37	4.42	4.47	4.12	0.11	0.39	0.18	0.08
Week 6	4.68	4.8	4.84	4.62	0.12	0.93	0.68	0.17

[§]Data are expressed as means (n=10). The main effects (the chemical form and concentration of supplemental methionine) were analyzed by two-way ANOVA and Duncan's multiple range test was used to compare the treatment means. Means in the same row without a common letter differ significantly ($P < 0.05$).

[‡]Photos of chicks were taken at weeks 5 and 6. Feather coverage was scored based on the photos on a scale of 1-5 with 1 being almost no feathering or less than 25% of the body covered, 2 being 25%-50% feather coverage, 3 being 50%-75% feather coverage, 4 being 75%-90% feather coverage and 5 being 100% feather coverage.

3.5 Discussion

The present study was performed to address the missing information in the literature on the comparative effects of two major supplemental methionine forms: DL-MET and HMTBA, at two concentrations in the grower and finisher diets of broilers on their metabolic responses to high ambient temperatures. To our best knowledge, this was the first of such attempts despite many past studies on the two forms of methionine supplements. One of the most interesting findings from our study was that supplementing 30% extra methionine in either form improved antioxidant status of broilers under such environmental condition. The improvements were manifested with enhanced GSH concentrations in the liver of the grower birds and elevated FRAP of the finishers. Although similar effects of supplemental methionine on tissue GSH concentrations were found by other groups in broiler[89-90] and quails[107], those animals were raised under normal temperatures. The observed benefits may be explained by the potential that methionine could be converted into cysteine for the synthesis of GSH[23-24]. Likewise, FRAP was improved by supplementing methionine and other antioxidant nutrients such as vitamins C and E to broilers[108-109]. Notably, the DL-MET supplementation produced higher GSH concentrations in the breast and thigh than the HMTBA supplementation in several instances.

In contrast, the extra methionine supplementation decreased SOD activity in the adipose tissue and GPx activity in the thigh of the growers and also decreased the GPx activity in the liver and GPx and GST activities in the adipose tissue in the finishers. Comparatively, the DL-MET-fed growers showed lower activities of various antioxidant enzymes in the assayed tissues than the HMTBA-fed birds. Seemingly, there was a methionine form - dependent effect on the tissue antioxidant defense responses to the high ambient temperature. Our previous study also indicated that supplementing diets with potent antioxidant astaxanthin for both broilers and laying hens under high ambient temperature decreased tissue activities of GPx, GR, and GST[76-77]. Supplementing broiler diets with methionine also decreased GPx activity in their liver and intestines[89]. Thus, there seemed to be a coordinated function or adaptive response between the intrinsic antioxidant enzyme production and the extrinsic antioxidant nutrient enrichment. Future research should be performed to assess this type of coordination in comparing the relative efficacy of different forms of methionine supplements in improving tissue antioxidant status of broilers. Furthermore, it is intriguing that the effects of supplemental methionine on the responses of GSH, FRAP, and antioxidant enzyme activities were not consistent across the assayed tissues, the two ages of birds, or the two forms and concentrations of methionine. While revealing underlying mechanisms for these variations or discrepancies may take a long time, we should currently develop a practical method to assess if those

sporadic enhancements of tissue GSH or antioxidant enzyme activities indeed contribute to the resistance of broilers to the high ambient temperature or other stressors.

Another interesting finding from our study was that the extra DL-MET supplementation elevated the hepatic gene expression of IL-6, AKT, and P38MAPK, while the extra HMTBA supplementation decreased the gene expression of IL-10 and HSP 70. Meanwhile, the hepatic mRNA levels of HSP 90 and JNK were higher in the DL-MET-fed groups than those in the HMTBA-fed groups. Pro-inflammatory cytokine IL-6 was previously found to be elevated under inflammatory status[63-64]. The elevated IL-6, along with ROS generated from oxidative stress, might up-regulate mitogen-activated protein kinases (MAPK) such as P38MAPK and JNK and subsequently might induce AKT to protect cells from oxidative injury or death[60, 65-66]. On the other hand, IL-10 and heat shock proteins (HSP70, HSP90) were reported to be up-regulated under oxidative stress to exert their anti-inflammatory effects[67-72]. Supplementing human subjects with antioxidant mixtures enhanced synthesis of heat shock proteins[110-111], which was somewhat similar to the elevated gene expression of HSP90 in the DL-MET-fed groups. In comparison, supplementing rats and human subjects with antioxidants decreased IL-6 but elevated IL-10 in blood[112-115]. Treating macrophage like cells (RAW 264.7) with natural anti-inflammatory products inhibited the activation of MAPKs[114-115]. These

results were in disagreement with our above-described findings. Although the chemical form and concentration of supplemental methionine in the diets for broilers indeed affected the hepatic inflammation-related gene expression, there is no simple interpretation to explain the observed mixed effects in the present study.

The third finding from the current study was that the extra methionine supplementation enhanced the concentrations of MUFA in the liver of growers. Because MUFA and PUFA are susceptible to oxidation and peroxidation, and supplemental antioxidants can prevent those destructions[75, 98], our finding provides evidence from a different angle for the antioxidant potential of supplemental methionine. The greater MUFA and PUFA concentrations in the liver of birds fed DL-MET than those fed HMTBA might also imply a better antioxidant efficacy of the DL-MET. However, as the high ambient temperature stress became more intensified from the grower to the finisher period, we observed opposite effects of the extra methionine supplementation and(or) DL-MET on fatty acid profiles in the breast and adipose tissue. It is relevant to note that lipid synthesis and activation of fatty acid synthase could relate to proinflammatory status[48], while breakdown of fatty acids might be related to anti-inflammatory phenotypes[48]. Previous chicken and pig studies have shown elevated lipid synthesis and storage under heat stress conditions[44-45, 47, 116-117]. Intriguingly, the decreased MUFA and PUFA concentrations in

the liver of the finishers fed the extra methionine or DL-MET might be associated with a relatively long term-improved antioxidant and anti-inflammatory status.

Compared with the commercial standards[106], the overall growth performances of birds during the starter and grower phases were similar, but impaired during the finisher phase by the high room temperature. Specifically, the body weight, average daily gain, and feed intake were 14, 13, and 22% lower than the commercial targets (Cobb-Vantress, 2018). The impairment was consistent with results reported by previous studies[11, 118-120]. As heat stress is known to decrease feed intake, the greater feed intake in the DL-MET-fed groups than that in the HMTBA-fed groups suggested a potential benefit of supplementing the former under high ambient temperature conditions. Likewise, benefits of supplemental methionine into the amino acid-deficient diets on growth performance were shown in broilers raised under thermoneutral conditions [33-35]. In the present study, the extra methionine supplementation-compromised growth performance compared with the commercial goals and the lack of difference from the normal level of supplementation may be related to the chronic high temperature employed, especially toward the end of the study when the room temperature was 12 ^{o}C higher than the suggested temperature. That might be too extreme for the extra methionine to compensate for the performance loss of the birds. Although the extra methionine supplementation indeed partially

ameliorated adverse effects of the high ambient temperature on health and antioxidant status of the birds, these ameliorations were not sufficient to affect growth performance.

In conclusion, supplementing 30% extra methionine, either as DL-MET or HMTBA, into the corn-soybean meal-based diets for the grower and finisher broilers raised in high ambient temperature affected their antioxidant status, the inflammation-related gene expression in the liver, and the fatty acid profiles in several tissues. However, the effects varied with the tissues and ages of the birds, the selected measures, and the forms and concentrations of the supplemental methionine. Future research should be performed to explore how coordinated adaptations of the intrinsic ROS scavengers and antioxidant enzymes affect functions and efficacy of supplemental DL-MET vs. HMTBA in helping broilers to cope with various environmental stresses.

CHAPTER FOUR

Conclusion

Broilers raised in stress conditions such as high stocking density and high ambient temperature are susceptible to oxidative stress which results in impaired performance and wellbeing. Supplementing methionine in the diets of broiler diets may relief the adverse effects caused by the stress conditions due to its antioxidant capacity. To investigate whether supplementing methionine truly benefits the broilers raised under high stocking density and high ambient temperature and also to compare the effects of two forms of methionine supplementation DL-MET and HMTBA, two studies were conducted. The first study that supplemented 100 or 130% DL-MET in diets of broilers under high stocking density found that the DL-MET supplementation improved the feed conversion efficiency, antioxidant status, fatty acid profiles, and increased hepatic mRNA level of HSP90 of the birds under high stocking density. The second study which supplemented 100 or 130% DL-MET or HMTBA to diets of broiler raised under high ambient temperature found that the methionine supplementation as either form affected antioxidant status and the activities of antioxidant enzymes. The 130% methionine supplementation resulted in higher tissue fatty acid concentrations during the grower phase while decreased tissue fatty acid concentrations in the finisher phase. The second

study also found that DL-MET supplementation led to higher GSH concentrations in the tissues while lower antioxidant enzyme activities. In conclusion, the extra methionine supplementation had positive effects on antioxidant status of broilers under high stocking density and high ambient temperature and improved feed conversion efficiency of broilers subjected to high stocking density. The results of these studies indicated that supplemental methionine can be beneficial to the performance and wellbeing of broilers raised under stress conditions. However, these studies also had some limitations. Firstly, these studies did not investigate the mechanism as how methionine exerted its antioxidant effects because neither the circulating methionine concentrations nor the products synthesized from methionine in the one carbon cycle such as S-adenosylmethionine, homocysteine, and cysteine were measured in the tissues especially in the liver. And the activities of enzymes involved in the one carbon cycle were not measured. Secondly, due to the lack of antibodies, only mRNA levels of the inflammation related genes were tested, while results on the protein levels could be more straightforward and convincing. Due to the facility limitation, the second study did not have control groups with broilers raised under thermal neutral conditions. This was the third limitation of these studies. Future studies should be done to investigate the mechanism of the antioxidant capacity of methionine in the broilers. The potential coordination between the intrinsic antioxidant enzymes and antioxidant production and its relationship with extrinsic antioxidant supplementation should also be further studied.

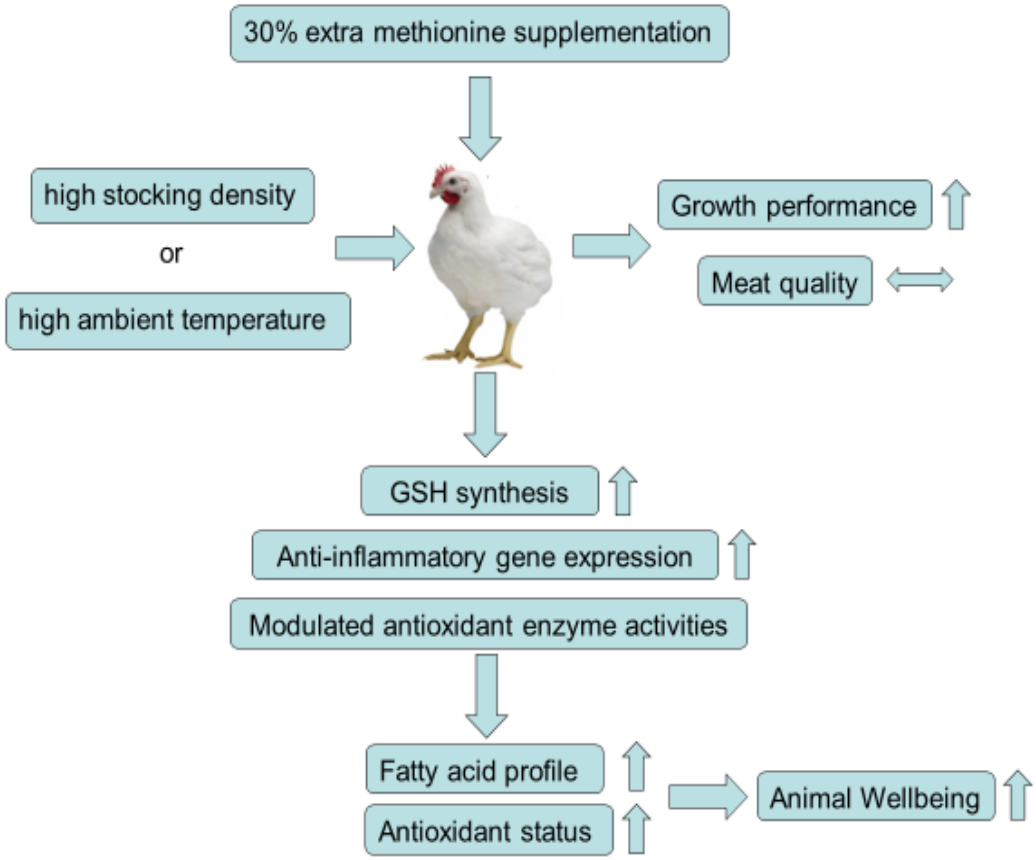

Figure 10. Effects of methionine supplementation on broilers raised under high stocking density or high ambient temperature. Supplementing 30% extra methionine on broilers under high stocking density or high ambient temperature did not affect the meat quality of the broiler but improved the growth performance of the broilers. The supplementation also elevated GSH synthesis, improved anti-inflammatory gene expression, and modulated the activities of antioxidant enzymes. These changes led to improved fatty acid profiles and antioxidant status and eventually better animal wellbeing.

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