# Effects of supplementation of DL-methionine on tissue and plasma antioxidant status during heat-induced oxidative stress in broilers

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**ABSTRACT** Exposure to high ambient temperature has been shown to impair growth performance and to cause oxidative stress in broilers. This study investigated the hypothesis that supplementation with methionine (**Met**) as DL-Met (**DLM**) more than the National Research Council recommendations improves growth performance and alleviates oxidative stress in broilers exposed to high ambient temperature. One-day-old male Cobb-500 broilers (n = 68) were allotted to 4 groups and phase-fed 3 basal diets during days 1 to 10, 11 to 21, and 22 to 35. One group was kept under thermoneutral temperature conditions and received the basal diets with Met + cysteine (**Cys**) concentrations according to recommendations of NRC. The other 3 groups were kept in a room with an increased ambient temperature from week 3 to 5 and were fed either the basal diet or the basal diets supplemented with 2 levels of DLM in which Met + Cys concentrations exceeded NRC recommendations by around 20% (group DLM1) and 40% (group DLM2), respectively. As expected, the broilers exposed to high ambient temperature showed a lower feed intake, lower body weight gains, a higher feed:gain ratio, and biochemical indications of oxidative stress in comparison to broilers kept under thermoneutral temperature conditions. Supplementation of DLM did not improve the growth performance in broilers exposed to high ambient temperature. However, the broilers supplemented with DLM had increased concentrations of glutathione in liver and breast muscle (groups DLM1 and DLM2), increased concentrations of tocopherols in the liver (group DLM2), and reduced concentrations of 7α-hydroxycholesterol and 7-ketocholesterol in heat-processed thigh muscle (groups DLM1 and DLM2) in comparison to the control group exposed to high ambient temperature. Concentrations of thiobarbituric acid-reactive substances and vitamin C in plasma, liver, and muscle were not different between the 3 groups exposed to heat stress. Nevertheless, the study shows that supplementation of DLM in slight excess of the Met concentration required for maximum growth performance improved the antioxidant status in tissues and reduced the susceptibility of muscle toward oxidation in heat-stressed broilers.

**Key words:** methionine, heat stress, oxidative stress, antioxidant response, broiler

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## INTRODUCTION

Methionine (**Met**) is commonly supplemented to broiler diets to meet requirements of sulfur-containing amino acids (**SAA**) for maximum growth performance. The function of Met is not limited to its role as a proteinogenic amino acid as it has several other functions in the body. One of these functions is its contribution to the antioxidant system (Brosnan and Brosnan, 2006). This function is explained by the fact that Met is a precursor of cysteine (**Cys**) (Ingenbleek and Kimura, 2013) which again acts as a precursor or constituent of substances such as taurine and glutathione (**GSH**) which are important antioxidants in the body (Mari et al., 2009; Jong et al., 2012). Antioxidants (e.g., tocopherol, vitamin C, GSH) and antioxidant enzymes (e.g., superoxide dismutase, catalase, glutathione peroxidase) are 2 important components of the body's antioxidant defense system which is needed to eliminate



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reactive oxygen species (**ROS**) which are also produced during normal metabolism (Lykkesfeldt and Svendsen, 2007). Externally added oxidants and compounds which either stimulate ROS production or weaken the antioxidant defense can disturb the balance between ROS generation and elimination, resulting in the development of oxidative stress (Lushchak, 2014). Under such conditions, oxidative damage of molecules like lipids is occurring, and generation of for example lipid peroxidation products such as thiobarbituric acid-reactive substances (**TBARS**) is induced (Lushchak, 2014).

Although it is already known that deficits of SAA compromise the antioxidant defense system (Bauchart-Thevret et al., 2009; Shen et al., 2015), the effect of Met supplementation is less clear when dietary SAA concentrations are close to concentrations which are recommended for maximal growth (Chen et al., 2014; Zeitz et al., 2017, 2018a). Additionally, because oxidative stress conditions increase consumption of antioxidants, the effect of Met supplementation may be modulated when animals are exposed to oxidative stress.

Chronic exposure to heat, a situation to which growing broilers are frequently exposed to worldwide, increases the production of ROS which leads to the depletion of antioxidants and in turn causes the development of oxidative stress (Akbarian et al., 2016). In such situations, higher supply of antioxidants or antioxidant precursors may increase the capacity of the cellular antioxidant defense and prevent oxidative damage (Kumbhar et al., 2018; Shakeri et al., 2018). Dietary supplementation of Met could be beneficial when broilers are exposed to heat because of an increased availability of Met metabolites for generation of antioxidants which are an important part of the antioxidant defense system. Therefore, this study investigated the hypothesis that supplementation of Met improves the antioxidant system in broilers exposed to high ambient temperature.

## MATERIALS AND METHODS

### *Birds, Husbandry, Diets, and Experimental Design*

A total of sixty-eight 1-day-old male broiler chickens (Cobb 500, Cobb, Wiedemar, Germany) were allotted to 4 experimental groups for a 5-wk feeding trial (5–6 broilers per cage and 17 broilers per group) and were subjected to different feeding and temperature conditions as explained below. The mean initial body weight (42.6 ± 2.5 g; mean ± SD) was similar among experimental groups. The broilers were kept in 2.1 $m^2$ cages equipped with nipple drinkers and feed automates and had free access to feed and water. They were kept on cardboards of which the upper crinkled cardboard layer was exchanged 2 times per week (day 1–14) or every 2 d (days 14–35). Light intensity was constantly at 40 Lux. The light regime was 24 h:0 h 23 h:1 h, 22 h:2 h, 21 h:3 h, 20 h:4 h, 19 h:5 h (light: dark) at days 1, 2, 3, 4, 5, 6, and 18 h:6 h from the seventh day on, similar to the recommendations of the breeder's guide. The room temperature decreased from 28°C–29°C to 27°C–28°C during the first 2 wk, and mean relative humidity was 61.1 ± 1.5%. During the first 6 d, infrared lamps (Albert Kerbl GmbH, Buchbach, Germany) were used as additional heat sources. From the third week on, different temperature conditions were applied. One experimental group was housed in a separate room and was kept under control (thermoneutral) temperature conditions and received control diets (CC group). The ingredient and nutrient composition of the 3 control diets fed during the starter (day 0–10), grower (day 11–21), and finisher phase (day 22–35) are shown in Table 1. In the CC group, the room temperature decreased from 27.3°C to 22.5°C on day 35, and mean relative humidity was 62.8 ± 1.4%. The other 3 experimental groups were kept in a temperature-controlled room and exposed to high temperature (moderate heat stress) that is the temperature was kept at 27.4°C ± 0.3°C, and mean relative humidity was 63.7 ± 0.6% until day 35. The heat-exposed birds were

**Table 1.** Ingredient and nutrient composition of the basal diets fed during the starter (day 1–10), grower (day 11–21), and finisher (day 22–35) period.[1]

| Item | Starter | Grower | Finisher |
|---|---|---|---|
| Ingredient (%) | | | |
| Maize | 50.0 | 54.9 | 58.8 |
| Soybean meal | 30.6 | 29.2 | 26.0 |
| Soybean oil | 2.76 | 3.60 | 4.16 |
| Maize gluten | 7.21 | 7.65 | 7.00 |
| Fish meal | 5.00 | - | - |
| Monocalciumphosphate | 1.43 | 1.53 | 1.24 |
| Limestone ($CaCO_3$) | 1.45 | 1.33 | 1.12 |
| Mineral and vitamin premix[2] | 1.00 | 1.00 | 1.00 |
| Salt (NaCl) | 0.24 | 0.36 | 0.33 |
| Choline Chloride | 0.10 | 0.12 | 0.13 |
| L-Lysine (54.6%) | 0.10 | 0.19 | 0.16 |
| L-Threonine (98.5%) | 0.05 | 0.06 | 0.05 |
| L-Valine (98.0%) | 0.03 | 0.03 | 0.01 |
| Nutrient composition (% as is)[3] | | | |
| DM | 89.0 (90.4) | 88.9 (88.1) | 88.9 (87.9) |
| Crude ash | 7.39 (7.12) | 6.85 (5.70) | 6.16 (6.22) |
| Crude fiber | 3.03 (3.58) | 3.06 (3.15) | 2.95 (3.40) |
| Crude fat | 6.09 (6.32) | 6.64 (5.48) | 7.25 (4.81) |
| Crude protein | 24.9 (24.8) | 21.6 (21.3) | 20.0 (19.8) |
| SID Lysine | 1.29 (1.23) | 1.10 (1.04) | 1.00 (0.98) |
| SID Methionine | 0.45 (0.45) | 0.38 (0.36) | 0.35 (0.35) |
| SID Methionine + Cysteine | 0.74 (0.73) | 0.65 (0.62) | 0.61 (0.61) |
| SID Threonine | 0.82 (0.82) | 0.71 (0.70) | 0.65 (0.66) |
| SID Tryptophane | 0.23 | 0.19 | 0.18 |
| SID Leucine | 2.07 (2.09) | 1.89 (1.87) | 1.77 (1.75) |
| SID Isoleucine | 0.90 (0.91) | 0.77 (0.78) | 0.71 (0.73) |
| SID Valine | 1.02 (1.02) | 0.88 (0.88) | 0.80 (0.82) |
| ME (MJ/kg) | 12.7 (12.5) | 13.0 (12.2) | 13.3 (12.4) |

[1]Starter and grower diets contained an anticoccidiostatic drug (Maxiban, 0.375 g/kg; on top).

[2]The premix supplied per kg diet: Ca, 3 g, Cl, 0.01 g, vitamin A, 12,000 IU, vitamin D3, 4,000 IU, vitamin K3, 3.33 mg, biotin, 250 µg, folic acid, 1.67 mg, vitamin B1, 3.33 mg, vitamin B2, 8 mg, Vitamin B6, 4.17 mg, vitamin B12, 25 µg, nicotinamide, 69.1 mg, calcium pantothenate, 20 mg, choline chloride, 400 mg, Fe, 50 mg, Cu, 15 mg, Mn, 100 mg, Zn, 70 mg, I, 1.56 mg, Se, 0.25 mg. Vitamin E was omitted in the premix.

[3]Values show calculated values based on AMINODat 5.0, and values in brackets are analyzed values. SID = standardized ileal digestible. Analyzed SID amino acids were calculated from analyzed amino acid concentrations with the help of amino acid digestibility values from AMINODat 5.0. The metabolizable energy (ME) content of the diet was calculated based on crude nutrient analyses of the dietary ingredients or for values in brackets of diets, according to GfE (Gesellschaft für Ernährungsphysiologie) (1999).

fed either the 3 control diets (HC group), or diets supplemented with DLM as a source of Met in 2 different concentrations (0.19 and 0.37% in starter diets, 0.16 and 0.32% in grower diets, 0.15 and 0.29% in finisher diets, Table 2). The Met + Cys concentrations of the control diets were in agreement with NRC (National Research Council, 1994) recommendations for broilers. However, standardized ileal digestible (**SID**) Met + Cys levels were about 15% lower than those recommended by the breeder (Cobb-Vantress Inc., 2015) and 20% lower than those recommended by AMINOChick 2.0 (Evonik Industries, Germany). The diets supplemented with the low or the high Met level exceeded the recommendations of NRC (1994) for Met + Cys by around 20 and 40%, respectively, the SID recommendations of the breeder for Met + Cys by around 2 to 6% or 17 to 26%, respectively and the recommendations of AMINOChick 2.0 (Evonik Industries, Essen, Germany) by 0% and 13 to 19%, respectively. Vitamin E was not supplemented via the premix to avoid masking of oxidative stress generation, but the recommendations were met according to estimated vitamin E concentrations of the dietary ingredients. All experimental procedures were in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS NO. 123, https://rm.coe.int/168007a445). The animals were killed by bleeding after prior electrical anesthesia. The regional council of Giessen approved the experiment (V54 - 19c 20 15 h 01 GI 19/3 Nr. G27/2017).

## Data Recording and Sample Collection

Body weight (individually) and feed intake (per cage) were determined on days 1, 10, 21, and 35, and the feed:gain ratio was calculated from feed intake and gains on cage basis. The mortality of the birds was recorded and considered for calculations. The respiration rate (breaths/min) was recorded twice daily from 2 randomly selected broilers per cage for 10 s per each broiler from week 3 onward. Feed samples were collected after feed manufacturing from each of the diets and were stored at −20°C.

At slaughter (day 35), the weights of carcass, breast muscle, thighs (including bones), and liver were recorded. Whole blood was collected in 10-mL tubes containing 100 µL of 10% EDTA solution. For later analysis of GSH and glutathione disulfide (**GSSG**) according to Guistarini et al. (2013), 200 µL of whole blood and 20 µL of 310 mM N-ethylmaleimide were combined, mixed, incubated for ≥1 min, and stored at −80°C. For normalization of GSH and GSSG to hemoglobin, hemoglobin concentrations were measured in whole blood according to the manufacturer's instructions with Drabkin's Reagent (#D5941, Sigma-Aldrich, Taufkirchen, Germany). Plasma was prepared by centrifugation at 1,100 × *g* at 4°C for 15 min and stored at −20°C. For later analysis of vitamin C, the plasma was processed according to Sato et al. (2010). In brief, 400 µL plasma and 400 µL 10% metaphosphoric acid (containing 1 mM ethylenediaminetetraacetic acid) (**MPA/EDTA**) were combined, vortexed, and centrifuged at 21,000 g for 10 min at 4°C. For analysis of vitamin C, 90 µL supernatant were combined with 410 µL of 5% MPA/EDTA, vortexed for 10 s, centrifuged at 20,000 g at 4°C, and the supernatant was stored at −80°C. For analysis of vitamin C, 90 µL supernatant were combined with 10 µL of 350 mM tris(2-carboxyethyl)phosphine and incubated on ice in the dark for 2 h. Afterward, 400 µL of 5% MPA/EDTA were added, vortexed for 10 s, centrifuged at 20,000 g at 4°C, and the supernatant was stored at −80°C.

Samples of liver, breast muscle, and thigh muscle were snap-frozen in liquid nitrogen and stored at −80°C for later analysis of triacylglycerols, TBARS, tocopherols, and vitamin C. For later analysis of GSH and GSSG, samples of liver and breast muscle (50 mg) were homogenized in 0.5 mL of 50 mM tris(hydroxymethyl)aminomethane (**TRIS**) with serine/boric acid/acivicin/N-ethyl maleimide, pH 8.0 for 3 min at 30 Hz in the TissueLyzer (Qiagen, Hilden, Germany) and acidified with 50 µL of 60% trichloroacetic acid before centrifugation for 2 min at 14,000 g and room temperature and storage of supernatant at −80°C according to Giustarini et al. (2013).

The left thigh (including skin and bones) was stored at −20°C in zip-lock bags for later heating (170°C for 50 min in a drying oven). Samples of the heated thigh muscle were collected and stored at −80°C pending analysis of TBARS and cholesterol oxidation products (**COP**).

## Laboratory Analyses

Feed samples were analyzed for DM, CP (N × 6.25), crude lipids, crude fiber, and crude ash by the methods 3.1., 4.1.1., 5.1.1, 6.1.1., and 8.1 of Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten (VDLUFA, 2013) and were analyzed for amino acids by using ion-exchange chromatography with postcolumn derivatization with ninhydrin (Association of Official Analytical Chemists (AOAC), 1995; European Commission, 2009). In brief, amino acids were oxidized with performic acid, which was neutralized with Na metabisulfite, and were then liberated from the protein by hydrolysis with 6 N hydrochloric acid for 24 h at 110°C and quantified with the internal standard by measuring the absorption of reaction products with ninhydrin at 570 nm. The concentration of tryptophan in feed raw components was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm) after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110°C (European Commission, 2000). The concentration of tyrosine was not determined.

For determination of vitamin C in muscle and liver, tissue homogenates were prepared with MPA and Tris(2-carboxyethyl)phosphine hydrochloride, and vitamin C was analyzed by HPLC-ECD (L-7100, LaChrom, Merck-Hitachi, Darmstadt, Germany) on a

Lichrospher 100 RP-18 column (5 µm; 250 × 4 mm; Merck) combined with an RP18 guard column according to Sato et al. (2010).

The concentrations of α- and γ-tocopherol in plasma and tissue homogenates were determined by HPLC (L-7100, LaChrom, Merck-Hitachi, Darmstadt, Germany) according to Zeitz et al. (2016). In the results section, the concentration of total tocopherols is reported which is calculated as the sum of the concentrations sum of α- and γ-tocopherol. The concentrations of TBARS were determined with a fluorescence spectrometer in plasma and in tissue homogenates prepared with TRIS hydrochloride buffer after methanolic sodium hydroxide extraction as described in Zeitz et al. (2018a) based on previously described methods (Wong et al., 1987; Khoschsorur et al., 2000). The concentrations of triacylglycerols in plasma and in tissue extracts, where lipids had been extracted with hexane-isopropanol (3:2, v/v) and dissolved in Triton X-100 (Eder and Kirchgessner, 1994; Zeitz et al., 2018b), were determined with a commercial kit (Fluitest TG, Analyticon). The concentrations of GSH and GSSG in whole blood and tissue homogenates were determined according to Giustarini et al. (2013) and Moore et al. (2013) by HPLC-MS.

The oxysterols 7α-hydroxy(OH)-, 7β-OH- and 7keto-cholesterol were extracted from thigh muscle tissue aliquots with 1 mL Folch-reagent (chloroform/methanol; 2:1; v:v) per 10 mg dried tissue as described in Schött et al. (2017). Butylated hydroxytoluene (50 µg added per mL solvent) was used as antioxidant and added before extraction. Extraction was performed overnight at 4°C in a dark cold room. The extracts were kept at −20°C until analysis. Oxysterol concentrations were analyzed by gas chromatography-mass spectrometry according to the procedure as described by Schött and Lütjohann (2015).

### Statistical Analysis

The statistical analysis of the data was performed by means of the statistical program packages BMDP (Dixon, 1993) and the Minitab statistical software (Rel. 13.1, Minitab, State College, PA). Data were checked for normal distribution. Normal distributed data are shown as arithmetic means and the SDs. If the distribution of a variable was skewed to the right, a logarithmic transformation was applied before all analyses were done, and data are shown as geometric mean and the dispersion factor (= geometric SD). The 2 control groups without or with heat stress (groups CC, HC) were compared by $t$ test for independent samples or the Wilcoxon-Mann-Whitney for data lacking normal distribution. For the 3 groups subjected to heat stress (groups HC, DLM1, DLM2), an analysis of variance (ONEWAY) with group as factor was performed. For statistically significant F values, means of the 3 groups were compared by Fisher's multiple range test. In all tests and comparisons of means a level of significance of $P = 0.05$ was used. To elucidate the reason for a reduced COP production in the heat-exposed broilers supplemented with DLM, correlations between concentrations of vitamin C, tocopherols, GSH in plasma (or blood), liver, and muscle, and the concentrations of COP (7α-OH-cholesterol, 7β-OH-cholesterol, 7-keto-cholesterol) in heat-processed thigh muscle were calculated within the 3 groups exposed to heat stress (groups HC, DLM1, DLM2).

## RESULTS

### Effect of Exposure to High Ambient Temperature on Broiler Performance, Physiological Stress, and Antioxidant Status

As expected, exposure to high ambient temperature affected bird performance unfavorably (Table 3). Final body weights, weight gains, feed intake, carcass weights, and proportion of breast meat were lower in the group fed the control diet subjected to high ambient temperature (HC group) than in the group fed the control diet kept under thermoneutral conditions (CC group, Table 3). The feed:gain ratio and the water intake were higher in the HC group than in the CC group (Table 3). The respiration rate of the broilers was higher in the HC group compared with the CC group in week 3 to 5 (Table 4). The antioxidant status was affected by exposure to heat in plasma and liver but not in breast muscle (Table 5). The HC group had a lower total tocopherol concentration in plasma and a higher concentration of GSSG and a lower GSH:GSSG ratio in blood than the CC group (Table 5). In the liver, the HC group showed a higher concentration of GSH and GSSG and lower concentrations of vitamin C and total tocopherols than the CC group (Table 5). Concentrations of TBARS, a marker for oxidative damage of tissue lipids, in plasma, liver, and muscle were similar in both groups of broilers (Table 5). Concentrations of COP—7α-OH-cholesterol, 7ß-OH-cholesterol and 7-keto-cholesterol—in heat-processed thigh muscle were higher in the HC group than in the CC group (Table 5).

**Table 2.** Concentrations of methionine and methionine and cysteine in the experimental diets (% as is).

| Item | Control | DLM1 | DLM2 |
|---|---|---|---|
| Starter | | | |
| Met | 0.50 (0.50) | 0.69 (0.65) | 0.87 (0.87) |
| Met + Cys | 0.87 (0.86) | 1.06 (1.02) | 1.24 (1.24) |
| Grower | | | |
| Met | 0.42 (0.40) | 0.58 (0.55) | 0.73 (0.72) |
| Met + Cys | 0.76 (0.73) | 0.92 (0.89) | 1.07 (1.06) |
| Finisher | | | |
| Met | 0.39 (0.39) | 0.54 (0.53) | 0.68 (0.69) |
| Met + Cys | 0.71 (0.71) | 0.86 (0.84) | 1.00 (1.01) |

Abbreviations: DLM, DL-Met; Met, methionine; M + C, methionine and cysteine.

Values show calculated values based on AMINODat 5.0, and values in brackets show analyzed values.

**Table 3.** Growth performance and carcass characteristics of broilers kept under control temperature conditions and fed a control diet (CC) or kept under high ambient temperature conditions and fed either a control diet (HC) or diets supplemented with either a low (DLM1) or a high (DLM2) supplementation level of DL-methionine.[1]

| | | High ambient temperature | | | |
|---|---|---|---|---|---|
| Item | CC | HC | DLM1 | DLM2 | ONEWAY (P)[2] |
| Performance data | | | | | |
| Initial body weight (g) | 42.7 ± 0.76 | 42.4 ± 0.87 | 42.5 ± 0.64 | 42.6 ± 0.72 | 0.947 |
| Final body weight (g) | 2,663 ± 108 | 2,436 ± 47[3] | 2,501 ± 179 | 2,542 ± 44 | 0.531 |
| Weight gain (g) | 2,620 ± 109 | 2,394 ± 48[3] | 2,459 ± 179 | 2,499 ± 45 | 0.534 |
| Feed intake (g) | 3,739 ± 121 | 3,482 ± 61[3] | 3,539 ± 223 | 3,634 ± 47 | 0.437 |
| Feed: gain ratio (g/g) | 1.43 ± 0.02 | 1.45 ± 0.01[3] | 1.44 ± 0.05 | 1.45 ± 0.01 | 0.797 |
| Water intake, day 11–day 35 (mL) | 6,114 ± 311 | 7,215 ± 59[3] | 7,141 ± 612 | 7,256 ± 278 | 0.938 |
| Carcass characteristics | | | | | |
| Eviscerated carcass weight (g) | 1,972 ± 188 | 1,845 ± 195 | 1,842 ± 284 | 1,878 ± 138 | 0.869 |
| Dressing percentage (%) | 74.1 ± 2.7 | 74.4 ± 2.5 | 73.5 ± 4.0 | 73.8 ± 2.3 | 0.676 |
| Breast muscle (% of body weight) | 23.2 ± 1.8 | 22.1 ± 1.9[3] | 22.5 ± 2.6 | 22.6 ± 1.8 | 0.785 |
| Thighs (% of body weight) | 19.5 ± 1.4 | 20.0 ± 1.3 | 19.9 ± 1.3 | 19.6 ± 0.9 | 0.574 |
| Liver (% of body weight) | 2.58 ± 0.19 | 2.48 ± 0.24 | 2.63 ± 0.46 | 2.58 ± 0.29 | 0.445 |

[1]Results are means ± SD, n = 3 for performance data, n = 15–17 for carcass characteristics data.
[2]ONEWAY was performed for the 3 high ambient temperature groups (HC, DLM1, DLM2).
[3]Significant difference between HC and CC group by *t* test for independent samples ($P \leq 0.05$).

## *Effect of DLM Supplementation on Broiler Performance, Physiological Stress, and Antioxidant Status*

The supplementation of DLM at 2 different levels in heat-exposed broilers had no effect on performance (final body weights, weight gains, feed intake, feed:gain ratios), water intake, and carcass characteristics in comparison to the heat-exposed broilers of the control group (Table 3). The respiration rate of the heat-exposed broilers in week 3 and 5 were not influenced by DLM supplementation (Table 4). However, in week 4, the group supplemented with the higher level of DLM (DLM2 group) had a lower respiration rate than the control group kept at high temperature (HC group, Table 4).

Supplementation of both levels of DLM in heat-exposed broilers caused an increase in GSH in liver and breast muscle to in comparison to heat-exposed broilers fed the control diet the HC group (Table 5). In the liver, the concentration of GSSG was also increased in the groups supplemented with DLM, whereas the GSH:GSSG ratio was not different in comparison to the heat-exposed control group (Table 5). The group supplemented with the higher level of DLM (DLM2 group) also had a higher concentration of total tocopherols in the liver and tended to have a higher concentration of total tocopherols in plasma ($P < 0.10$) than the heat-exposed control group (HC group) (Table 5). Concentrations of TBARS and vitamin C in plasma, liver, and breast muscle were not significantly different between heat-exposed broilers supplemented with DLM (DLM1, DLM2 groups) and those fed the control diet (HC group, Table 5). However, the groups supplemented with DLM (DLM1, DLM2) tended to have lower concentration of TBARS in the liver ($P < 0.10$) than the heat-exposed control group (Table 5). Moreover, the groups supplemented with both levels of DLM (DLM1, DLM2 groups) had lower concentrations of 7α-OH-cholesterol and 7-keto-cholesterol in heat-processed thigh muscle than the heat-exposed group fed the control diet (HC group, Table 5). The concentration of 7β-OH-cholesterol was not different between the heat-exposed broilers supplemented with DLM (DLM1, DLM2 groups) and those fed the control diet (HC group, Table 5).

Correlations between concentrations of antioxidants in plasma (or blood), liver, and muscle and the concentrations of COP in heat-processed thigh muscle are shown in Table 6. There were negative correlations between GSH concentration in the liver and the concentrations of 7α-OH-cholesterol and 7-keto-cholesterol. There

**Table 4.** Respiration rate of broilers kept under control temperature conditions and fed a control diet (CC) or kept under high ambient temperature conditions and fed either a control diet (HC) or diets supplemented with either a low (DLM1) or a high (DLM2) supplementation level of DL-methionine.[1]

| | | High ambient temperature | | | |
|---|---|---|---|---|---|
| Item | CC | HC | DLM1 | DLM2 | ONEWAY (P)[2] |
| Respiration rate (breaths/min) | | | | | |
| Week 3 | 74.1 ± 0.8 | 87.7 ± 2.8[3] | 83.0 ± 3.6 | 86.0 ± 2.2 | 0.210 |
| Week 4 | 57.4 ± 2.1 | 84.3 ± 0.7[3,a] | 82.4 ± 0.1[b] | 82.9 ± 0.8[b] | 0.021 |
| Week 5 | 54.0 ± 0.4 | 91.1 ± 2.2[3] | 90.4 ± 5.3 | 91.4 ± 1.7 | 0.930 |

[a,b]Means with different superscript letters within the 3 groups with high ambient temperature differ significantly ($P \leq 0.05$).
[1]Results are means ± SD, n = 3.
[2]ONEWAY was performed for the 3 high ambient temperature groups (HC, DLM1, DLM2).
[3]Significant difference between HC and CC group by *t* test for independent samples ($P \leq 0.05$).

**Table 5.** Concentrations of antioxidants and oxidation products in plasma, blood, liver, breast muscle, and heated thigh muscle of broilers kept under control temperature conditions and fed a control diet (CC) or kept under high ambient temperature conditions and fed either a control diet (HC) or diets supplemented with either a low (DLM1) or a high (DLM2) supplementation level of DL-methionine.[1]

| | | High ambient temperature | | | |
|---|---|---|---|---|---|
| Item[2] | CC | HC | DLM1 | DLM2 | ONEWAY (P)[3] |
| Plasma | | | | | |
| Vitamin C (µmol/l) | 66.3 ± 6.8 | 69.7 ± 17.5 | 60.7 ± 13.2 | 63.0 ± 7.8 | 0.189 |
| Total tocopherols (mmol/mol TAG)[4] | 25.9 ± 8.0 | 20.1 ± 5.3[6] | 18.9 ± 5.2 | 24.9 ± 9.8 | 0.069 |
| TBARS (mmol/mol TAG) | 17.1 ± 6.9 | 18.5 ± 7.2 | 21.0 ± 12.2 | 17.4 ± 6.8 | 0.541 |
| Blood | | | | | |
| GSH (µmol/g Hb) | 16.3 ± 1.9 | 17.5 ± 2.7 | 18.4 ± 2.2 | 19.0 ± 2.1 | 0.234 |
| GSSG (nmol/g Hb) | 5.66 ± 1.06 | 9.05 ± 2.32[6] | 10.3 ± 3.1 | 8.86 ± 2.51 | 0.297 |
| GSH:GSSG ratio | 2,964 ± 574 | 2,017 ± 417[6] | 1,909 ± 540 | 2,278 ± 598 | 0.160 |
| Liver | | | | | |
| Vitamin C (µmol/g) | 2.14 ± 0.18 | 1.93 ± 0.19[6] | 1.89 ± 0.18 | 1.87 ± 0.18 | 0.623 |
| GSH (µmol/g) | 2.58 ± 0.44 | 3.04 ± 0.52[6,b] | 4.08 ± 0.49[a] | 4.17 ± 0.44[a] | <0.001 |
| GSSG (nmol/g) | 4.58 ± 1.40 | 5.58 ± 1.78[b] | 8.19 ± 3.60[a] | 8.50 ± 2.54[a] | 0.010 |
| GSH:GSSG ratio | 603 ± 180 | 582 ± 140 | 575 ± 196 | 524 ± 132 | 0.557 |
| Total tocopherols (nmol/g)[4] | 29.0 ± 4.5 | 23.3 ± 3.6[6,b] | 25.9 ± 5.0[a,b] | 28.2 ± 5.9[b] | 0.031 |
| TBARS (nmol/g) | 45.8 ± 11.7 | 44.9 ± 9.1 | 36.6 ± 11.6 | 36.0 ± 12.3 | 0.060 |
| Breast muscle | | | | | |
| Vitamin C (nmol/g) | 233 ± 41 | 225 ± 42 | 209 ± 37 | 198 ± 24 | 0.123 |
| GSH (µmol/g) | 1.20 ± 0.53 | 1.22 ± 0.43[b] | 1.63 ± 0.35[a] | 1.75 ± 0.33[a] | 0.001 |
| Total tocopherols (nmol/g)[4] | 13.4 ± 2.7 | 13.2 ± 2.6 | 12.8 ± 2.7 | 12.6 ± 1.6 | 0.736 |
| TBARS (nmol/g)[5] | 3.21; 2.13 | 2.81; 1.89 | 2.17; 2.59 | 2.25; 2.25 | 0.935 |
| Thigh muscle, heated | | | | | |
| TBARS (nmol/g) | 92.9 ± 17.6 | 91.4 ± 30.0 | 90.0 ± 30.0 | 83.3 ± 13.0 | 0.494 |
| 7α-OH-cholesterol (µmol/mol cholesterol) | 245 ± 125 | 513 ± 274[6,a] | 268 ± 77[b] | 308 ± 92[b] | 0.001 |
| 7ß-OH-cholesterol (µmol/mol cholesterol) | 367 ± 123 | 538 ± 218[6] | 440 ± 137 | 489 ± 129 | 0.312 |
| 7-keto-cholesterol (µmol/mol cholesterol)[5] | 324; 1.44 | 619; 1.68[6,a] | 398; 1.32[b] | 455; 1.28[b] | 0.003 |

[a,b]Means with different superscript letters within the 3 groups with high ambient temperature differ significantly ($P \leq 0.05$).
[1]Results are means ± SD, n = 15.
[2]TBARS = thiobarbituric acid-reactive substances, GSH = reduced glutathione, GSSG = oxidized glutathione.
[3]ONEWAY was performed for the 3 high ambient temperature groups (HC, DLM1, DLM2).
[4]Total tocopherols = α-tocopherol + γ-tocopherol.
[5]Data were not normal distributed. Results are given as geometric means and dispersion factors.
[6]Significant difference between HC and CC group by *t* test for independent samples ($P \leq 0.05$).

was also a negative correlation between plasma tocopherol concentration and the concentration of 7β-OH-cholesterol, and there were tendencies toward negative correlations between plasma tocopherol concentration and the concentrations of 7α-OH-cholesterol and 7-keto-cholesterol ($P < 0.10$). There were moreover significant positive correlations between vitamin C plasma concentration and concentrations of 7α-OH-cholesterol, 7β-OH-cholesterol, and 7-keto-cholesterol and significant positive correlations between muscle vitamin C concentration and the concentrations of 7α-OH-cholesterol and 7-keto-cholesterol.

## DISCUSSION

The present study was performed to investigate the hypothesis that supplementation of DLM alleviates changes of the antioxidant system caused by heat stress

**Table 6.** Correlations (r) between concentrations of GSH, tocopherols, and vitamin C in plasma (or blood), liver, muscle and concentrations of 7α-OH-cholesterol, 7β-OH-cholesterol and 7-keto-cholesterol in heat-processed thigh muscle within the 3 groups of broilers subjected to heat stress (*P*-values are shown in parentheses).

| Item | 7α-OH-cholesterol | 7β-OH-cholesterol | 7-Keto-cholesterol |
|---|---|---|---|
| GSH blood | 0.023 (0.896) | 0.070 (0.686) | 0.010 (0.953) |
| GSH liver | −0.532[1] (0.001) | −0.265 (0.113) | −0.493[1] (0.002) |
| GSH muscle | −0.045 (0.791) | 0.174 (0.303) | −0.033 (0.848) |
| Tocopherols plasma | −0.309 (0.067) | −0.383[1] (0.021) | −0.299 (0.077) |
| Tocopherols liver | −0.162 (0.332) | −0.083 (0.622) | −0.147 (0.378) |
| Tocopherols muscle | 0.078 (0.640) | −0.023 (0.893) | 0.062 (0.711) |
| Vitamin C plasma | 0.456[1] (0.005) | 0.327[1] (0.048) | 0.461[1] (0.004) |
| Vitamin C liver | 0.196 (0.232) | 0.093 (0.572) | 0.168 (0.307) |
| Vitamin C muscle | 0.392[1] (0.015) | 0.202 (0.224) | 0.407[1] (0.011) |

Abbreviation: GSH, glutathione.
[1]Indicates a statistically significant correlation ($P < 0.05$), n = 45.

in broilers. It has been shown that environmental temperatures higher than 25°C elicit heat stress in poultry (Shakeri et al., 2020). The temperature of 27.4°C maintained in week 3 to 5 of the trial is slightly higher than this critical temperature, suggesting that the broilers in this study suffered only from moderate heat stress. Under practical conditions, during seasonal hot periods, ambient temperatures can reach much higher levels, causing a more pronounced heat stress to broilers than in the present study.

To give an assessment of the antioxidant status, we determined concentrations of antioxidants (GSH, vitamin C, total tocopherols) and oxidation products (TBARS) in blood or plasma, respectively, liver and muscle. Moreover, we determined the concentrations of 3 major COP (7α-OH-cholesterol, 7β-OH-cholesterol, 7-keto-cholesterol) in heat-processed thigh muscle. The concentration of COP in animal products are low, but their formation is enhanced during storage or heat-processing from cholesterol in a nonenzymatic reaction by the action of ROS occurring in the cell (Eder et al., 2005; Kim et al., 2006). Therefore, the concentrations of COP are regarded as specific indicators of oxidative stress, and their concentrations in heat-processed animal tissues reflect their susceptibility toward oxidation (Eder et al., 2005; Maldonado-Pereira et al., 2018).

In agreement with several other studies (Sahin et al., 2016; He et al., 2018; Lu et al., 2018), we observed that broilers exposed to high temperature show a reduced feed intake, reduced body weight gains, and an increased feed:gain ratio in comparison to the broilers kept under thermoneutral temperature condition. The reduction in feed intake observed in heat-stressed animals is an attempt to cope with heat stress by reducing the metabolic heat production (Mujahid et al., 2005). Recently, it has been observed that heat stress influences the secretion or gene expression of appetite-related hormones and genes which might be linked to the reduction of feed intake (He et al., 2017). We also observed a decreased proportion of breast muscle in broilers exposed to high ambient temperature, a common finding in broilers exposed to heat which might be because of a reduced muscle protein synthesis and an increased muscle protein decomposition (Lu et al., 2018; Ma et al., 2018). An increased respiration rate observed in the heat-exposed broilers in week 3 to 5 is another typical symptom observed in broilers under heat stress, which aims to dissipate heat from the body to the environment (Wiernusz and Teeter, 1995). Moreover, we observed an increased consumption of water in the birds kept under high ambient temperature which is a typical adaptation in birds subjected to heat stress to control the body temperature (Sayed and Downing, 2015).

The Met + Cys concentration in the control diet used in this study was in agreement with NRC recommendations for broilers (National Research Council, 1994), but SID Met + Cys was around 20% below the recommendations given by industry and 15% below the breeder. We found that supplementation of this diet with 2 different levels of DLM, yielding dietary Met + Cys concentrations which were in excess to the requirement defined under thermoneutral conditions, did not improve feed intake and growth of heat-exposed broilers and also did not influence carcass characteristics, including proportion of breast muscle. This finding indicates that Met was not growth-limiting in the control diet used under conditions of heat stress. The finding that DLM supplementation did not increase growth performance under heat stress condition agrees with some other studies which also showed that Met in excess of the requirement for maximum growth is not able to improve growth in heat-stressed broilers (Ribeiro et al., 2005; Willemsen et al., 2011; Liu et al., 2019). Interestingly, Balnave and Oliva (1990) which performed dose–response relationship trials under different temperature environments observed that the Met requirement for maximum growth of broilers is even lower under high temperature condition than under thermoneutral condition. While DLM supplementation had no effect on growth performance of the heat-exposed broilers in this study, both groups supplemented with DLM showed a lower respiration rate in week 4 than the control group. However, as this effect was not observed in week 3 and 5, it is unlikely that supplementation of DLM improved the adaptation of broilers to heat stress.

In agreement with several other studies, we observed that exposure to high ambient temperature induces oxidative stress in broilers (Altan et al., 2003; Huang et al., 2015; Sahin et al., 2017; Wen et al., 2019). Oxidative stress in broilers subjected to heat stress was evident by a reduction of the concentrations of vitamin C and tocopherols in the liver, an increase of GSSG concentration and a reduction of the GSH:GSSG ratio in blood and an increase of the GSH concentration in the liver. Depletion of antioxidants such as tocopherols and vitamin C is a hallmark of oxidative stress because elevated concentrations of ROS lead to an increased consumption of these antioxidants (Sahin et al., 2003; Eder et al., 2005). The lower concentration of vitamin C, an antioxidant which is endogenously synthesized in broilers, may be also explained by a reduced synthesis under heat stress (Khan et al., 2012). Higher GSH concentrations in heat-exposed birds may indicate that GSH synthesis was upregulated which is known to happen under conditions of oxidative stress (Lu, 2009) and has already been shown in heat-stressed broilers (Willemsen et al., 2011). The increased GSSG concentration and the reduced GSH:GSSG ratio observed in blood of heat-stressed broilers are indicative of increased oxidation of GSH. We also observed that broilers subjected to high ambient temperature fed the control diet had higher concentrations of COP (7α-OH-cholesterol, 7ß-OH-cholesterol, 7-keto-cholesterol) in heat-processed thigh muscle than broilers fed the control diet kept under thermoneutral temperature condition. This finding is another indication that broilers kept under high ambient temperature were subjected to an increased level of oxidative stress in comparison to the broilers kept under thermoneutral temperature condition.

We observed that under these oxidative stress conditions in broilers exposed to high ambient temperature, supplementation of DLM caused an increase of the concentrations of GSH in liver and breast muscle. This observation is in agreement with Zeitz et al. (2018a), where increased levels of liver GSH were also observed when dietary Met + Cys was supplemented above growth requirements in thermoneutral conditions. GSH, one of the major antioxidant compounds in the body, is a tripeptide consisting of Cys, glutamate, and glycine. Cysteine can be synthesized from Met by the trans-sulfuration pathway (Ingenbleeck and Kimura, 2013). The finding of an increased GSH concentration suggests that an increased Met availability due to DLM supplementation enhanced the channeling of Met into the trans-sulfuration pathway to form Cys for GSH synthesis. We moreover observed a significantly increased concentration of total tocopherols in the liver and a tendency toward an increased concentration of total tocopherols in plasma of broilers supplemented with the higher level of DLM (group DLM2). It is well known that tissue tocopherol concentrations are lowered by the induction of oxidative stress, because of an increased consumption of tocopherols in the course of lipid peroxidation (Eder et al., 2005). Thus, the finding that tocopherol concentrations were increased in the DLM2 group is another indication that supplementation of DLM could attenuate oxidative stress in heat-stressed broilers. An increase of tocopherols concentrations in plasma and liver by DLM supplementation was also observed under thermoneutral conditions (Zeitz et al., 2018a). We moreover observed that heat-stressed broilers supplemented with the 2 levels of DL-Met had lower concentrations of 7α-OH-cholesterol and 7-keto-cholesterol in heat-processed thigh muscle than control broilers. The COP, including 7α-OH-cholesterol and 7-keto-cholesterol, are not only regarded as specific indicators of oxidative stress, but they are also relevant with respect to the nutritive quality of broiler muscle as a food. The COP from foods are absorbed in the intestine, enter the liver, are incorporated into lipoproteins, and reach all tissues of the body (Emanuel et al., 1991; Linseisen and Wolfram, 1998). In cells, they are predominantly incorporated into membranes, where they affect important membrane properties such as their structure and fluidity and the activities of membrane-bound enzymes. The COP also promote the development of several diseases such as cardiovascular diseases and cancer (Kulig et al., 2016). Thus, a reduction of the concentrations of COP in animal products might be considered as beneficial with respect to human health. The finding that there were strong negative correlations between the GSH concentration in the liver and the concentration of 7α-OH-cholesterol and 7-keto-cholesterol indicates that the reduction of COP formation in heat-processed thigh muscle of broilers supplemented with DLM might have been at least in part because of an increased GSH production. The observation that there was also a significant correlation between plasma tocopherol concentration and the concentration of 7β-OH-cholesterol (and tendencies between plasma tocopherol concentration and the concentration of 7α-OH-cholesterol and 7-keto-cholesterol) suggests that an improved vitamin E-status contributed also to reduced COP concentrations in the broilers supplemented with DLM. An unexpected finding was that that there were positive correlations between vitamin C concentrations in plasma and muscle and the COP formation in heat-processed thigh muscle. It would have been expected that vitamin C as an antioxidant should be able to prevent the formation of COP. There is indeed a study showing that supplementation of vitamin C to beef patties prevents the formation of COP during deep frying (Wong and Wang, 2013). However, in another study, vitamin C supplementation enhanced the formation of COP in chicken meat (Grau et al., 2001). In guinea pigs fed a diet with oxidized fats, vitamin C supplementation had no effect on the formation of COP in vivo (Keller et al., 2004). Overall, the role of vitamin C in COP formation remains unclear.

In contrast to concentrations of COP, there were only minor effects of DLM supplementation on concentrations of TBARS in tissues. The only effect of DLM supplementation on TBARS concentration was a tendency toward a reduction in the liver while TBARS concentrations in plasma and muscle remained unaffected. The TBARS are commonly determined in studies dealing with oxidative stress as an indicator of lipid peroxidation, mainly because of the fact that they can be simply assayed. However, it should be noted that TBARS are a very unspecific parameter of oxidative stress as thiobarbituric acid reacts with a variety of aldehydes and the breakdown products of proteins and carbohydrates (Ghani et al., 2017). Thus, the finding that TBARS concentration remained largely unchanged must not necessarily be contradictory to the suggestion that supplementation of DLM caused an improvement of the antioxidant status but could be because of the low specificity of the TBARS assay. Other studies have similarly reported that while no significant changes were observed for oxidative stress parameters such as malondialdehyde, a marker of lipid peroxidation, a significant increase could be observed for antioxidant markers like ferric reducing ability of plasma when higher levels of Met + Cys were fed (Liu et al., 2019). We observed that the concentrations of vitamin C in plasma and tissues, in contrast to concentrations of total tocopherols, remained unchanged by DLM supplementation in broilers exposed to high ambient temperature. This finding agrees with recent studies in turkeys in which Met supplementation caused an improvement of the antioxidant status as indicated by the antioxidant capacity and concentrations of oxidation products in tissues, while concentrations of vitamin C in plasma, liver, and muscle remained unchanged (Jankowski et al., 2017a,b).

Some studies have already been published dealing with effects of Met supplementation on the antioxidant system in various animal species. However, in most of them the Met concentration in control diet was below

the requirements for optimum growth. A general finding in these studies, which is in agreement with the present study, was that supplementation of Met to a Met insufficient diet increases the concentrations of GSH in various tissues, which has been observed in broilers, heat-stressed quails, piglets, or turkeys (Chen et al., 2014; Del Vesco et al., 2014; Jankowski et al., 2017a; Zeitz et al., 2017; Liu et al., 2019). Some of the studies also found an increase of the activities of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, or catalase in the liver (Del Vesco et al., 2014; Jankowski et al., 2017a; Liu et al., 2019) and a reduction of the concentrations of oxidation products such as TBARS or protein carbonyls in plasma (Jankowski et al., 2017a; Zeitz et al., 2017). These findings indicate that supplementation of Met to a diet with an insufficient Met concentration improve the antioxidant status in broilers and other species. However, it has been shown that an increase of the dietary Met concentration in excess of the requirement does not further improve the antioxidant status in broilers which were not subjected to diet or heat stress (Zeitz et al., 2017). This finding is in opposite to the finding of the present study which showed that supplementation of DLM to a diet which was already sufficient in Met (according to growth performance parameters) led to an improvement of the antioxidant status in heat-exposed broilers. It is likely that this difference is attributed to the fact that heat stress compromises the antioxidant system, and more Met is required to counteract the development of oxidative stress. Recently, it has been observed that supplemental Met lowers the expression of HSP90, a member of the heat shock proteins in the liver of broilers. The authors concluded that the decrease of this heat shock protein could reflect on improvement of the antioxidant system (Magnuson et al., 2020). Heat shock proteins are also upregulated during heat stress periods. They stimulate cytoprotection and induce heat tolerance in the cells of various organs and tissues by repairing damaged or misfolded proteins and promoting cell survival (Hasan Siddiqui et al., 2020). It would be interesting to elucidate whether supplementation of Met is also able to influence the expression of heat shock proteins in tissues broilers of during exposure to heat stress. It has been shown that heat stress in broilers leads to immunosuppression, particularly by an impairment of the humoral immunity, which can increase the risk of secondary infections (Bartlett et al., 2003; Quinteiro-Filho et al., 2010). In this respect, it would be interesting to find out whether an improvement of the antioxidant system in heat-stressed broilers by supplementation of Met could improve the immune system and thus reduce the risk of infectious diseases.

In the present study, we used 2 different supplementation levels of DL-Met to elucidate the optimum dietary concentration of Met to attenuate oxidative stress in heat stress-exposed broilers. We observed that the first level yielding a total Met concentration of the diet slightly in excess of the requirement for optimum growth caused already a maximum increase of GSH concentration in tissues and a maximum decrease of concentrations of 7α-OH-cholesterol and 7-keto-cholesterol in heat-processed thigh muscle.

## CONCLUSION

The present study shows that supplementation of DLM more than the requirement for maximum growth does not alleviate the adverse effects of exposure to high ambient temperature on growth performance in broilers. As expected, exposure to high ambient temperature caused oxidative stress in liver and muscle tissue of broilers. Supplementation with DLM increased the concentrations of GSH in liver and muscle and reduced the concentration of 7α-OH-cholesterol and 7-keto cholesterol, 2 of the major COP, in heat-processed thigh muscle. These findings indicate that supplementation of DLM in slight excess of the requirement for optimum growth improved of the antioxidant status in liver and muscle and reduced the susceptibility of the muscle toward oxidation in broilers exposed to heat stress.

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