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Non Ruminant Nutrition

NON RUMINANT NUTRITION

Supplemental methionine and stocking density affect antioxidant status, fatty acid profiles, and growth performance of broiler chickens

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Abstract

Broilers stocked in high densities may be prone to oxidative and inflammatory insults, resulting in impaired health status, growth performance, and meat quality. This study was to determine if 30% extra supplemental DL-methionine alleviated or prevented those adverse effects of a higher stocking density in broiler chickens. A total of 560 male Cornish Cross cockerels (day old) were divided into four groups: two stocking densities (9 and 12 birds/m^2) and two supplementations of methionine (grower: 2.90 or 3.77 g/kg and finisher: 2.60 or 3.38 g/kg). Growth performance was recorded weekly. Blood and tissues were sampled at the end of each period. High stocking density decreased ($P < 0.05$) body weight and growth performance of growers and (or) finishers. Those differences were partially attenuated by the extra methionine supplementation. The high methionine elevated ($P < 0.05$) glutathione (**GSH**) concentration in the thigh at both ages (> 24%). The high stocking density elevated (>28%, $P < 0.05$) glutathione concentration in the plasma, breast, and thigh of growers, but decreased ($P < 0.05$) it in the liver of growers and thigh of finishers. Interaction effects ($P < 0.05$) between dietary methionine and stocking density were found on activities of the antioxidant enzyme glutathione S-transferase in the liver of growers and breast, thigh, and adipose tissue of finishers. The interaction effect was also found on activities of glutathione peroxidase and superoxide dismutase in the thigh of growers. The extra methionine decreased ($P < 0.05$) hepatic gene expression of heat shock protein 90 (18%) and thigh and breast malondialdehyde concentrations of the finishers (35%). In conclusion, the 30% extra DL-methionine supplementation was able to partially mitigate adverse effects caused by the higher stocking density and to improve the redox status of the broilers.

Key words: antioxidant, broiler, methionine, oxidative stress, stocking density

Introduction

Modern broiler houses are designed to raise the animal flocks at relatively high densities to save space and revenue. 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 (Proudfoot et al., 1979; Dafwang et al., 1987; Shanawany, 1988; Dozier et al., 2005; Houshmand et al., 2012). Reactive oxidative

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Abbreviations

ADG	average daily gain
AKP	alkaline phosphatase
AKT	protein kinase b
ALT	alanine aminotransferase
AME	apparent metabolizable energy
BW	body weight
DL-MET	DL-methionine
FI	feed intake
FRAP	ferric reducing ability of plasma
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione S-transferase
HSP70	heat shock protein 70
HSP90	heat shock protein 90
IL-10	interleukin 10
IL-6	interleukin 6
JNK	c-Jun N-terminal kinase
MDA	malondialdehyde
MUFA	monounsaturated fatty acid
NEFA	nonesterified fatty acid
P38MAPK	P38 mitogen-activated protein kinase
PIP	inorganic phosphorus
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SFA	saturated fatty acids
SOD	superoxide dismutase
TC	total cholesterol
TEP	1,1,3,3-tetraethoxypropane
TG	triglyceride
TNFα	tumor necrosis factor α
WHC	water holding capacity

species (**ROS**) are byproducts of energy metabolism in tissues of animals and can be readily elevated by environmental stresses such as crowding. Oxidative stress impairs the health and growth performance of broilers (Simitzis et al., 2012; Li et al., 2019). Dietary supplementations of antioxidant nutrients or compounds that can neutralize ROS and reduce oxidative stress are known to improve health and performance of animals under those stress conditions (Fellenberg and Speisky, 2006; Chen et al., 2013; Oroian and Escriche, 2015; Niu et al., 2018).

Sulfur-containing amino acids such as cysteine and methionine are capable of scavenging ROS (Atmaca, 2004). Thus, methionine not only is the first-limiting amino acid in chicken diets for protein synthesis but also plays an important role in antioxidation. Supplementing dietary methionine beyond requirements has been shown to improve antioxidant status (Chen et al., 2013) and immune function (Swain and Johri, 2000; Rama Rao et al., 2003) of growing broilers. The sulfur group in methionine is readily oxidized into sulfoxide under oxidative conditions, which enables methionine to scavenge ROS and protect other amino acids with essential functions from oxidation (Levine et al., 1996,2000; Atmaca, 2004). Methionine is also involved in the one-carbon cycle (Miller, 2003). In the cycle, methionine can be converted into cysteine which is the precursor of glutathione (**GSH**), a potent antioxidant (Miller, 2003).

Although previous studies indicated that health and growth performance of broilers were impaired by high stocking density-mediated oxidative stress (Levine et al., 1996, 2000; Rama Rao et al., 2003; Chen et al., 2013), there has been no published report on the potential of supplemental extra methionine in broiler diets in alleviating such negative effects. Therefore, we performed the present study and housed broiler chickens at a normal or a higher stocking density and fed them corn–soybean meal-based grower and finisher diets containing 100% or 130% recommended supplemental synthetic methionine (as **DL-MET**) for 6 wk. Our hypothesis was that the 30% extra supplemental DL-MET might help overcome the anticipated negative effects of higher stocking density on the physiological status and growth performance of broilers. Our objectives were to determine if the stocking density and the extra supplemental DL-MET affected: 1) growth performance, health, antioxidant, and inflammatory status, meat quality, feather coverage, and bone strength and 2) lipid and fatty acid profiles, antioxidant status, and expressions of inflammation-related genes.

Materials and Methods

The protocol was approved by the Cornell University Institutional Animal Care and Use Committee (Protocol number: 2010-0106). The experiment was conducted at the Large Animal Research and Teaching Unit, Cornell University, Ithaca, NY.

Animal, diets, and management

A total of 560 Cornish Cross cockerels purchased from Moyer's Chicks, Quakertown, PA, and were randomly divided into four treatment groups (10 pens per group) in a 2 × 2 factorial arrangement with two stocking densities and two supplemental DL-MET levels. Chickens were raised in floor pens (1 by 1 m) in environmentally controlled rooms with 2:22 (D:L) h cycles with free access to water and feed. According to the industry guide (Cobb-Vantress, 2018), the room temperature was initially set at 33 °C on day 0, and then was decreased by 3 °C every week to 18 °C that was maintained until the end of the study. Room humidity was maintained between 30% and 40%. The normal stocking density was also referred to the same industry guide (Cobb-Vantress, 2018), and the high stocking density was allotted according to a previous study (Dozier et al., 2006). At the beginning of the study, each of the 1 m^2 floor pens housed 12 birds for the normal density and 16 birds for the high density. At the end of the grower phase, the total birds/pen were decreased to 9 and 12 for the normal and high densities, respectively, by euthanizing and culling three and four birds per pen for sample collection.

All the birds were fed the same corn and soybean meal basal diet (crumbled) during the starter period (days 0 to 10). During the grower (days 11 to 22) and finisher (days 23 to 42) periods, birds were fed experimental pelleted diets supplemented with different concentrations of methionine (>99% pure DL-MET product, MetAMINO Evonik Industries, Essen, Germany). Specifically, 2.90 and 2.60 g DL-MET was supplemented per kilogram of diets for the growers and finishers, respectively, to meet 100% of the nutrient requirement of broilers based on AMINOChick 2.0 (Evonik Nutrition & Care GmbH, Germany). Meanwhile, a 30% extra of supplemental DL-MET (3.77 and 3.38 g of DL-MET/kg of diets for the growers and finishers, respectively) was fed to birds for potential benefits to attenuate the stocking density-related stress without apparent risk of overdosing (Lemme et al., 2002; Hoehler et al., 2005). The diets were formulated to meet all other nutrient requirements of the broilers and nutrient compositions of the diets are shown in Table 1.

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[1]	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[2]	5.00	5.00	5.00	5.00	5.00
L-Lysine	4.50	3.60	3.60	3.20	3.20
DL-Methionine, >99%[3]	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					
AME, kcal/kg	2,927	2,977	2,987	3,033	3,082
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[4], %	1.06	0.91	0.91	0.76	0.76

[1]Analytical nutrient values of corn: AME, 3,320 kcal/kg; crude protein, 77.5 g/kg; lysine, 2.51 g/kg; methionine, 1.64 g/kg. Analytical nutrient values of soybean mean: AME, 2,370 kcal/kg; crude protein, 474 g/kg; lysine, 29.3 g/kg; methionine, 66.3 g/kg.
[2]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, 1,575 mg; Cu, 12 mg; I, 0.053 mg; Mn, 30.2 mg; Se, 0.09 mg; Zn, 53.0 mg; Fe,67.8 mg.
[3]DL-Methionine (MetAMINO Evonik Industries, Essen, Germany, >99% purity).
[4]The calcium levels listed in the table are the calculated concentrations.

Measures and sample collection

Body weight (**BW**) and feed intake (**FI**) were recorded weekly to measure growth performance. Mortality was recorded daily. At the end of the grower and finisher periods, two birds from each pen were euthanized for sample collection. Plasma, liver, adipose tissue, breast, thigh, and tibia were collected from each bird following the methods described in our previous study (Liu et al., 2019). The actual sample collection time from multiple animals of different treatments were rotated and fully balanced.

Plasma and tissue biochemical and nutritional indicators

Activities of alanine aminotransferase and alkaline phosphatase (**AKP**) in the plasma and the concentrations of plasma inorganic phosphorus, glucose, total cholesterol (**TC**), triglycerides (**TG**), nonesterified fatty acid (**NEFA**), and uric acid were analyzed following the methods described in previous studies (Magnuson et al., 2018; Sun et al., 2018). The lipid profiles (TC, TG, and NEFA) were measured in the liver, adipose tissue, breast, and thigh using methods as described in previous studies (Magnuson et al., 2018; Sun et al., 2018). Fatty acids were extracted and methylated (Christie and Han, 2010) and were quantified using a gas chromatography (Agilent 6890N, Agilent Technologies, Santa Clara, CA) fitted with a flame ionization detector and a fused silica capillary column coated with CP-SIL 88 for fatty acid methyl esters (100 m × 0.25 mm i.d., 0.22 mm film thickness, Varian Inc., Lake Forest, CA) as described previously(Magnuson et al., 2018).

Plasma and tissue antioxidant status

Concentrations of total GSH, glutathione disulfide (**GSSG**) in the plasma, liver, breast, and thigh, and concentrations of malondialdehyde (**MDA**) in the liver, adipose, breast, and thigh were assayed using methods adapted from previous studies (Magnuson et al., 2018; Sun et al., 2018). Previously described methods were used to determine the ferric reducing ability of plasma (**FRAP**) (Benzie and Strain, 1996) and concentrations of protein carbonyl in the liver (Levine et al., 1990). Commercial kits (Cayman Chemical, Ann Arbor, MI) were used to measure plasma corticosterone and amyloid A concentrations. Activities of glutathione peroxidase (**GPx**), glutathione S-transferase (**GST**), glutathione reductase (**GR**), and superoxide dismutase (**SOD**) were measured in the breast, thigh, liver, and adipose tissue using methods adapted from the previous studies (Massey and Williams, 1965; McCord and Fridovich, 1969; Mannervik and Guthenberg, 1981; Flohée and Güunzler, 1984).

Quantitative real-time PCR

Abundances of interlukin-6, interlukin-10, tumor necrosis factor alpha , heat shock protein 70 (**HSP70**), heat shock protein 90 (**HSP90**), protein kinase B, P38 mitogen-activated protein kinases, and c-Jun N-terminal kinase mRNA in the liver were determined. Primers used for these tested genes are listed in Supplementary Table S1. Total mRNA was isolated and purified from the liver using TRIzol Reagent (Life Technologies, Carlsbad, CA) following the established method (Chomczynski and Sacchi, 1987). The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY) was used to perform

the mRNA reverse transcription. The real-time qPCR (7900 HT; Applied Biosystems) and the $2^{-\text{delta delta Ct}}$ ($\Delta\Delta$Ct) equation (Livak and Schmittgen, 2001) were used to quantify the mRNA levels.

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

The meat quality measurements included pH and water holding capacity (**WHC**), and texture profile analysis was done following the previously established methods (Sun et al., 2018). 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 (Turner and Burr, 1993; Gatrell et al., 2017). Severities of woody breast and white striping and feather coverage were evaluated by the scoring system adopted from the previous study (Liu et al., 2019).

Statistical analysis

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

Results

Growth performance and plasma biochemical indicators

There were no interaction effects of the stocking density and dietary methionine on any of the growth performance measures at any time point. During the starter phase, all the groups of chicks were fed the same corn and soybean meal basal diet with recommended DL-MET supplementation while two stocking densities were allotted to these animals. In fact, the stocking density showed effects ($P < 0.01$) on FI and feed/gain (Table 2). The BW of both growers and finishers in the high stocking density groups were lower ($P < 0.05$) than that in normal density groups. The average daily gain (**ADG**) of the growers and the overall ADG were decreased ($P < 0.05$) by the high stocking density in the 100% DL-MET groups, but not in the 130% DL-MET groups. FI was decreased ($P < 0.01$) by the high stocking density in all three phases. The birds in the high stocking density groups had better ($P < 0.05$) feed to gain ratio than those in the normal density groups in the starter and finisher phases. The 130% DL-MET diet decreased ($P < 0.05$) FI of the finishers compared with the 100% DL-MET diet.

The high stocking density did not affect the plasma health indicators or lipid profiles except for an elevated ($P < 0.05$) TG of the growers (Table 3). The 130% DL-MET diet enhanced ($P < 0.01$) plasma TC concentration and AKP activity of the growers and plasma uric acid and TG concentrations of the finisher broilers compared with the 100% DL-MET diet. Plasma concentrations of glucose and TG of the growers were decreased by the 130% DL-MET diet. An interaction effect ($P < 0.05$) of the stocking density and DL-MET concentration was observed in the grower phase on plasma AKP.

Tissue lipid and fatty acid profiles

The lipid profiles in the breast, thigh, or liver were not affected by the stocking density or the concentration of DL-MET supplementation (Table 4). In the finishers, the high stocking 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 (Table 5). The 130% DL-MET diet elevated ($P < 0.05$) the concentrations of total fatty acids and MUFA in the liver of the finishers, while decreased ($P < 0.05$) the concentration of MUFA in the breast of the growers. 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 growers.

Table 2. Effect of different stocking densities and concentrations of DL-methionine supplementation on growth performance of broilers[1]

Density		Normal		High			P-value		
Methionine	Period	100%	130%	100%	130%	SEM	Density	MET	Interaction
BW, g/chick	Starter[2]	345^{a}	336^{ab}	332^{b}	336^{ab}	2.14	0.13	0.52	0.12
	Grower	1,203^{a}	1,187^{ab}	1,152^{b}	1,180^{ab}	24.3	0.04	0.66	0.13
	Finisher	2,956^{a}	2,907^{ab}	2,832^{b}	2,854^{ab}	83.1	0.03	0.72	0.36
ADG, g/chick/d	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
	Overall	67.8^{a}	66.7^{ab}	64.9^{b}	65.4^{ab}	0.89	0.03	0.72	0.36
FI, g/chick/d	Starter	42.9^{a}	41.8^{a}	37.5^{b}	36.6^{b}	0.57	<0.01	0.09	0.85
	Grower	107^{a}	105^{ab}	100^{c}	101^{bc}	1.63	<0.01	0.84	0.33
	Finisher	180^{a}	169^{b}	164^{bc}	160^{c}	4.73	<0.01	0.01	0.32
	Overall	122^{a}	118^{ab}	117^{b}	115^{b}	1.65	0.02	0.06	0.53
Feed/Gain	Starter	1.59^{a}	1.56^{a}	1.42^{a}	1.38^{b}	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
	Finisher	1.75^{a}	1.68^{ab}	1.67^{ab}	1.62^{b}	0.03	0.03	0.06	0.67
	Overall	1.76	1.72	1.76	1.71	0.02	0.77	0.70	0.78

[1]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 ($P < 0.05$).

[2]During the starter phase, all the four groups of chickens were fed the same corn and soybean meal basal diet with recommended DL-MET supplementation while different stocking densities were applied to these groups.

Table 3. Effect of different stocking densities and concentrations of DL-methionine supplementation on plasma indicators of broilers[1]

Density	Normal		High			P-value		
Methionine	100%	130%	100%	130%	SEM	Density	MET	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[b]	287[b]	251[b]	455[a]	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

[1]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 ($P < 0.05$).

Plasma and tissue antioxidant status

The high stocking 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 6). In the finishers, the high stocking density decreased ($P < 0.01$) the concentrations of GSSG in the plasma, liver, and breast and the concentration of GSH in the thigh. The 130% DL-MET diet decreased ($P < 0.05$) the concentration of GSSG in the plasma but enhanced both GSH and GSSG in the thigh of the growers compared with the 100% DL-MET diet. There was an interaction effect ($P < 0.05$) of density and DL-MET in the finisher broilers: the 130% DL-MET diet elevated plasma GSH in the normal density groups whereas there was no effect of DL-MET in the high stocking density groups. An interaction effect was also observed on the concentration of MDA in the breast which was highest ($P < 0.05$) in the normal density group fed 100% DL-MET compared with the other three treatment groups. The 130% DL-MET diet decreased ($P < 0.05$) the concentrations of MDA in the thigh and breast. There were no effects of treatment on plasma FRAP, serum amyloid A, corticosterone, or protein carbonyl concentrations.

The high stocking density decreased ($P < 0.05$) the activity of GST in the thigh of both grower and finisher birds (Table 7). The activity of GR in the liver of the growers was decreased ($P < 0.05$) by the high stocking density. The 130% DL-MET diet 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, but elevated ($P < 0.05$) the activity of GR in the thigh of the finishers. Interaction effects ($P < 0.05$) of the stocking density and the DL-MET concentration were found on the activities of GPx and SOD in the thigh and GST in the liver of the growers. The 130% DL-MET diet elevated ($P < 0.05$) the activity of SOD under the high stocking density but did not affect the activity under the normal stocking density. The activity of hepatic GST was decreased ($P < 0.05$) by the high stocking density in the 130% DL-MET diet groups, but not the 100% DL-MET diet 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 ($P < 0.05$) by the 130% DL-MET diet only under the high stocking density. In contrast, the activity of GST in adipose tissue was elevated ($P < 0.05$) by the 130% DL-MET diet only under the normal stocking density.

Inflammation-related gene expressions

The mRNA levels of the analyzed inflammation-related genes were not affected by either the stocking density or the concentration of DL-MET supplementation except for the mRNA levels of HSP90 that were lower ($P < 0.05$) in the 130% DL-MET diet groups than those in the 100% DL-MET diet groups (Table 8).

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

The high stocking density decreased ($P < 0.05$) WHC of the breast muscle, but elevated ($P < 0.01$) the springiness of the breast and thigh and hardness of the thigh (Supplementary Table S2). Compared with the 100% DL-MET diet, the 130% DL-MET diet decreased ($P < 0.01$) the WHC of the breast, but elevated ($P < 0.05$) the hardness of the breast and springiness of the thigh. Both the high stocking density and the 130% DL-MET diet elevated ($P < 0.05$) the maximum load of the tibia of the

Table 4. Effect of different stocking densities and concentrations of DL-methionine supplementation on tissue lipid profile of broilers[1]

Density	Normal		High			P-value		
Methionine	100%	130%	100%	130%	SEM	Density	MET	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, μmol/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	24.5	1.53	0.62	0.82	0.65
NEFA, μmol/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, μmol/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, μmol/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, μmol/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, μmol/g protein	32.6	38.3	37.8	43.7	4.28	0.09	0.07	0.98

[1]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 ($P < 0.05$).

growers (Supplementary Table S3). The treatments did not affect breast myopathy scores, mortality rate, or feather coverage scores (Supplementary Tables S4 and S5).

Discussion

Our study offers both physiological and molecular evidences for the potential role and mechanism of 30% extra DL-MET supplementation in the grower and finisher diets for broilers in coping with a moderate stocking density challenge. As expected, the elevated stocking density adversely affected the growth performance of broilers (Proudfoot et al., 1979; Dozier et al., 2005). The extra DL-MET supplementation seemed to help partially offset the adverse effects, although the remedy was not consistent across all phases or measures. It is useful to recognize that the higher stocking density employed in the present study was probably insufficient to produce drastic adverse effects on growth performance or physiology of broilers. Because the stocking density was on the final BWs of finisher broilers, starters or growers unlikely experienced or suffered from crowding. In fact, the high stocking density produced a 27% greater maximum load of tibia in the growers fed the 30% extra DL-MET diet, indicating a mixed effect of the stocking density by age. In addition, effects of methionine on body protein synthesis and intestinal mucosal growth (Bortoluzzi et al., 2019) might be accumulative, and the subsequent impacts on growth performance could be more readily shown in the finisher than the grower birds.

An interaction between the stocking density and 30% extra DL-MET was observed in grower plasma AKP activity, an indicator of enhanced bone synthesis or turnover (Atkins et al., 2011). This may help explain the increased maximum load of tibia of growers. It remains unclear to us why the trend of these increases in plasma AKP activity and maximum tibial load did not carry over to the finisher phase, except for a speculated reason of lowering mobility of finishers (Thomas et al., 2004). Previous studies have demonstrated that extra DL-MET and stocking density elevated plasma AKP activities (Singh et al., 2015; Kang et al., 2016).

Overall, the stocking density and the DL-MET supplementation showed only moderate effects on the fatty acid profiles, including total FA, saturated fatty acids, MUFA, and PUFA in the present study. The high stocking density decreased the total FA, MUFA, and PUFA in the breast and MUFA in the adipose tissue of the finishers without affecting FA profiles of growers. This decrease in FA concentration in finishers, but not growers, may be due to their exposure to relatively different stocking density stress severities over age with their body size. The extra MET supplementation deceased MUFA in the breast of growers and increased hepatic total FA and MUFA of finishers. These type of responses were similar to our previous observations from supplemental MET in diets of broilers raised at high ambient temperature (Liu et al., 2019), but different from results reported from studies supplementing diets with antioxidants for broilers raised at conventional conditions (Wood et al., 2004; Niu et al., 2018). Interestingly, an interaction between DL-MET and stocking density was observed on the breast tissue PUFA content of growers. This may be explained in part by the ability of methionine to enhance lipid catabolism via promoting

Table 5. Effect of different stocking densities and concentrations of DL-methionine supplementation on tissue fatty acid concentrations of broilers[1]

Density	Normal		High			P-value		
Methionine	100%	130%	100%	130%	SEM	Density	MET	Interaction
Fatty acids, mg/g tissue								
Liver								
Total								
Grower	15.0	15.5	14.9	16.4	2.05	0.66	0.85	0.82
Finisher	28.0	31.2	26.5	32.6	2.30	1.00	0.05	0.51
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
Finisher	7.34^{ab}	8.75^{ab}	6.18^{b}	10.3^{a}	1.19	0.87	0.02	0.23
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	8.23	7.00	0.36	0.95	0.08	0.07
Finisher	9.41^{a}	9.59^{a}	8.74^{ab}	7.40^{b}	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
Finisher	3.28^{a}	3.22^{a}	3.07^{a}	2.44^{b}	0.19	0.02	0.09	0.15
PUFA								
Grower	2.30	2.48	2.61	2.20	0.14	0.91	0.35	0.03
Finisher	3.02^{ab}	3.27^{a}	2.85^{ab}	2.49^{b}	0.18	0.01	0.74	0.09
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
Finisher	30.4^{a}	28.7^{a}	26.7^{ab}	20.4^{b}	2.64	0.04	0.16	0.41
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

[1]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 ($P < 0.05$).

carnitine synthesis which is potentially modulated by stocking density stress (Nukreaw et al., 2011). Likewise, these two treatments exerted mixed effects on the meat quality. The 30% extra DL-MET diet enhanced meat to bone ratio and springiness of thigh but decreased WHC of the breast in the finishers. This diet enhanced hardness of the breast in those at the normal stocking density, but not those raised at the high stocking density. Future research will need to determine

Table 6. Effect of different stocking densities and concentrations of DL-methionine supplementation on antioxidant status of broilers[1]

Density	Normal		High			*P*-value		
Methionine	100%	130%	100%	130%	SEM	Density	MET	Interaction
Grower								
Plasma, nmol/mL								
GSH	8.16^{b}	5.97^{b}	9.90^{a}	10.5^{a}	2.56	<0.01	0.25	0.19
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								
GSH	0.38^{b}	0.37^{b}	0.47^{a}	0.49^{a}	0.04	<0.01	0.16	0.51
GSSG	0.030	0.027	0.030	0.033	0.008	0.40	0.99	0.43
Thigh, μmol/g								
GSH	0.43^{c}	0.54^{b}	0.48^{bc}	0.65^{a}	0.06	<0.01	<0.01	0.29
GSSG	0.064^{ab}	0.073^{a}	0.052^{b}	0.068^{ab}	0.016	0.15	0.03	0.53
Finisher								
Plasma, nmol/mL								
GSH	7.92^{b}	11.5^{a}	10.3^{ab}	10.3^{ab}	1.87	0.57	0.05	0.03
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
Plasma FRAP, mmol Trolox equivalent/L								
	10.1	10.7	10.4	9.1	0.77	0.66	0.45	0.23
Plasma amyloid A, ng/mL								
	90.9	89.2	92.8	85.2	11.6	0.93	0.69	0.80
Plasma corticosterone, ng/mL								
	364	405	345	396	47.7	0.81	0.38	0.93
Liver protein carbonyl, nmol/mg protein								
	3.88	3.85	3.73	4.42	0.36	0.57	0.34	0.33

[1]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 ($P < 0.05$).

if these mixed changes in the mechanical characteristics of meat affect actual cooking properties and human sensory evaluation.

Both the high stocking density and the extra DL-MET supplementation appeared to elevate GSH concentrations in the plasma, liver, breast, and thigh of the broilers. Comparatively, the impact of the high stocking density on GSH was stronger and more consistent in the growers than in the finishers, whereas that of the extra DL-MET supplementation was somewhat contrary. These GSH change patterns in the growers in comparison within the finishers suggest other consequences or mechanisms of the stocking density aside from the crowding-induced oxidation or antioxidant responses. The GSH concentrations in the thigh showed the most consistent responses to the two types of treatments among the assayed tissues. Notably, hepatic GSH concentrations in the growers were decreased by the high stocking density, in contrast to the elevations in the plasma, breast, and thigh of the growers. Plausibly, this was because that GSH is synthesized mainly in the liver and transported into different tissues (Lu, 2000; Simitzis et al., 2012), the stocking density might have mediated a larger or quicker mobilization and transport of hepatic GSH to extrahepatic tissues than the hepatic synthesis of GSH. Although previous studies reported stress-induced (Akbarian et al., 2016) and extra dietary methionine-derived (Zhang et al., 2018) elevations of tissue GSH concentrations, our study has shown age- and tissue-dependent responses. It was also intriguing that the high stocking density displayed a trend to decrease tissue GSSG concentrations, in particular in the finishers. However, the dietary DL-MET supplementation exhibited only moderate impact or interaction with the stocking density on tissue GSSG concentrations. The extra DL-MET supplementation led to substantial decreases of MDA concentrations in the breast and thigh of the finishers. This attenuated tissue lipid peroxidation was in line with the

Table 7. Effect of different stocking densities and concentrations of DL-methionine supplementation on antioxidant enzymes activity of broilers[1]

Density	Normal		High			P-value		
Methionine	100%	130%	100%	130%	SEM	Density	MET	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
SOD	0.39^{ab}	0.36^{ab}	0.46^{a}	0.23^{b}	0.03	0.64	0.04	0.12
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
GST	68.6^{a}	68.5^{a}	69.1^{a}	51.1^{b}	2.07	0.02	0.01	0.02
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

[1]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 ($P < 0.05$).

effects of supplementing vitamin E, selenium, and astaxanthin into broiler diets (Leskovec et al., 2018; Niu et al., 2018).

The stocking density and dietary DL-MET supplementation also exerted mixed impacts on tissue activities of antioxidant enzymes. When broilers were fed the 100% DL-MET diet, the activities of GST in the adipose tissue of the growers and GR in the thigh of the finishers were elevated by the high stocking density. Interestingly, such elevations were removed by the extra DL-MET supplementation. Meanwhile, the activities of GST in the liver, thigh and adipose tissue of the finisher were decreased by the 130% DL-MET diet under the high stocking density. Elevated activities of antioxidant enzymes induced by stress were considered to be protective responses against oxidative stress (Davies, 1995; Altan et al., 2003). Reciprocally, decreased intrinsic activities of antioxidant enzymes in tissues were produced by supplementations of broiler diets with astaxanthin or methionine (Magnuson et al., 2018; Sun et al., 2018; Liu et al., 2019), indicating upregulated intrinsic antioxidant capacity. However, as shown in our previous study on the responses of antioxidant enzymes to a high ambient temperature and dietary MET supplementation (Liu et al., 2019), there were many inconsistent or opposite changes of these enzyme activities in the present study. Therefore, future research will need to explore the reason and mechanism for these variations.

It was somewhat surprising that the stocking density and the dietary MET concentration showed no effect on the hepatic

Table 8. Effect of different stocking densities and concentrations of DL-methionine supplementations on liver inflammation-related gene expressions of finisher broilers[1]

Density	Normal		High			P-value		
Methionine	100%	130%	100%	130%	SEM	Density	MET	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

[1]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 ($P < 0.05$).

mRNA levels of all eight assayed genes related to inflammation and stress with the exception of HSP90. The 130% DL-MET diet decreased the hepatic mRNA level of HSP90 (~20%) than the 100% DL-MET diet. Because gene expression of HSP90 was reported to be upregulated by stressors (Parsell and Lindquist, 1993; Kalmar and Greensmith, 2009), its decrease in the present study might reflect an attenuated oxidative status due to the extra methionine supplementation. This was also in agreement with previous findings associated with supplementing diets with vitamin E and selenium for broilers under high ambient temperature (Michailidis et al., 2013). However, the lack of the high stocking density to alter mRNA levels of the other assayed genes was contrary to their expected responses to stresses in other studies (Verhasselt et al., 1998; Cantley, 2002; Volonte et al., 2015; Zhang et al., 2016). Furthermore, the high stocking density failed to alter major oxidative stress-related biomarkers, including serum amyloid A, plasma FRAP and corticosterone, and liver protein carbonyl. Seemingly, the stocking density stress employed in the present study was not sufficiently high to induce the aforementioned changes or to allow a better chance for the benefit of the extra DL-MET supplementation. Due to the lack of antibodies, we could not tell if their protein productions were altered despite the absence of their mRNA level change.

In conclusion, our study revealed that both the elevated stocking density and the 30% extra DL-MET in the corn–soybean meal-based diets for the grower and finisher broilers exerted moderate effects on their growth performance, antioxidant, inflammatory, health status, lipid and fatty acid profiles, and meat quality. Some of the adverse effects derived from the stocking density could be partially attenuated or prevented by the 30% extra DL-MET supplementation. However, singular or interaction effects of these two types of treatments were fairly inconsistent. Future research may need to choose stronger stocking density stress than the level used in the present study and explore fundamental mechanisms of MET metabolism including proofing metabolites of methionine such as S-adenosylmethionine, homocysteine, and cysteine and characterizing functions of key enzymes involved in the circulation and tissues.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of interest statement

R.W. is an employee of Evonik Nutrition & Care GmbH, Hanau, Germany. All other authors declare no conflicts of interest.

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