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Effect of replacement different methionine levels and sources with betaine on blood metabolites, breast muscle morphology and immune response in heat-stressed broiler chickens

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ABSTRACT

This study was performed to evaluate the effect of replacement different methionine (Met) levels and sources (DL or L) with betaine on blood metabolites, breast muscle morphology and immune response in heat-stressed broiler chickens. A total of 1200 unsexed day-old Ross-308 chicks were raised under the same condition in the first 10 days and then reared under normal or subnormal (32 °C) ambient temperatures for the 11 to 42 days of age. The experiment was designed as a split-plot factorial arrangement with 2 (temperatures) × 2 (Met sources) × 3 (Met levels) × 2 (betaine amounts). Met level in the basal diet was 30% lower than recommendation (Low-Met) and was increased to recommendation (Rec-Met) or 30% more than it (High-Met) by supplemental DL- or L-Met. Betaine was or was not substituted at the rate of 30% of the supplemental DL- or L-Met. From 11 to 24 d of age, broilers fed high-met diets showed better FCR than those received Low- and Rec-Met diet. High-Met diet under heat stress (HS) showed highest plasma uric acid and homocysteine concentration than two other diets, under normal or HS condition. Replacing 30% of the supplemental Met with betaine showed lower plasma homocysteine concentration compared to non-supplemented betaine diets. Birds fed Low- and Rec-Met diets under normal condition showed a significant decrease in heterophil/lymphocyte ratio compared to their counterparts under HS. Birds fed L-Met supplemented diet showed a greater myofibers diameter than birds fed DL-Met diet. In general, High-Met diet decreased heterophil/lymphocyte ratio and FCR of broilers. A total of 30% of dietary supplemental Met can be replaced by betaine.

HIGHLIGHTS

- Heat stress increase methionine requirements of broiler chickens.
- L-methionine increases breast yield in compare with DL-methionine.
- Betaine in animal feed can be replaced by methionine without adverse effect on broiler chickens.

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Broiler chicken; heat stress; immune response; methyl donor; muscle fibre

Introduction

High ambient temperature causes a decrease in protein and amino acid digestibility (Yodseranee and Bunchasak 2012). This condition could enhance broiler demand to extra amino acid to synthesis of proteins or other specific compound like hormones and Hsp70 that can ameliorate the negative effect of heat stress (Reeds and Jahoor 2001). Methionine can also be catabolised to cysteine via the transmethylation-transsulfuration pathway and produced GSH that ameliorate the effects of reactive oxygen species (ROS) associated with high environmental temperature (Swennen et al. 2011). Thus, high methionine or total sulphur amino acids (TSAA) consumption is required for better performance of broiler chickens (Bunchasak 2009).

Met is mostly provided as DL-Met (99% purity power), which contains 50% L-Met and 50% D-Met. L-Met is considered the reference standard because only the L isomer of Met is deposited in the muscles or incorporated into enzymes. Since there are unique enzymatic pathway to convert Met isomers and analogs to L-Met in the liver and kidney (Baker 2006; Thwaites and Anderson 2007), the birds are able to use the isomers and analogs of Met for protein synthesis.

Uric acid, as the main end product of nitrogen metabolism, is indicative of amino acid requirements of broilers or the efficiency of amino acid utilisation (Donsbough et al. 2010; Zhai et al. 2016). Some studies have shown that plasma uric acid concentrations increase with increasing dietary nitrogen intake

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(Featherston 1969; Okumura and Tasaki 1969; Hevia and Clifford 1977). However, contradictory results have been reported when using plasma uric acid as a response variable to assess amino acid utilisation. In Xie et al. (2004) study, the ducklings' plasma uric acid content was decreased and then increased as dietary Met level was increased. Uric acid can serve as a hydroxyl radical scavenger (Carro et al. 2010) and inactivate an oxidant before they can react with biological molecules such as DNA, proteins, and lipid membranes (Sautin and Johnson 2008).

Homocysteine is formed as part of Met metabolism and may undergo irreversible transsulfuration to Cys or remethylation to Met by Met synthase or betaine–homocysteine methyltransferase (BHMT) (Pillai et al. 2006). The methyl group provided by this cycle is derived from betaine. Betaine, as the organic osmolyte and methyl donor, can be used to Met replacement in some important physiological processes such as protein and fat metabolism (Fernandes et al. 2009). A small portion of the supplemental betaine is metabolised to transmethylate homocysteine, while most of it is absorbed by tissues (Lever et al. 2004) that increase lean mass and decrease fat mass in pigs (Rojas-Cano et al. 2011) and chickens (Xing et al. 2011). The accumulation of betaine in cells during metabolic stress conditions increased the osmolality of the sarcoplasm, which helps to increase muscle mass (Cholewa et al. 2014).

The muscle fibre is a major component of skeletal muscle tissue. According to Damez and Clerjon (2008) findings, the number, size, and type of muscle fibre, as well as their as well as histological, biochemical, and biophysical characteristics of muscle fibres, may lead to changes in meat quality. It has been shown that betaine has many effects on muscle growth under metabolic and nutritional stress conditions (Fernandez-Figares et al. 2002; Cholewa et al. 2014).

Recognising and studying on broiler chicken muscle histological characteristics is one of the most important goals of the poultry scientists. Therefore, the present study aimed to evaluate the effect of replacement different Met levels and sources with betaine on blood metabolites, breast muscle morphology and immune response in heat-stressed broiler chickens.

Materials and methods

Experimental design, diets, and birds

A total of 1200 unsexed one-d-old Ross 308 broiler chicks were obtained from a local commercial hatchery and used in this experiment to compare two dietary supplemental Met sources and betaine replacement. The chicks were reared in 1.2 m × 1 m floor pens on 6 cm of wood shavings into two poultry houses with similar conditions except for extra heating system to create HS induction, as the main-plot and 12 diets as the sub-plot, with 5 replicates of 10 birds each (initial body weight, 42 ± 1.2 g). The trial was conducted as a split-plot factorial arrangement of 2 × 2 × 3 × 2 (temperature × Met source × Met level × betaine replacements on added Met, respectively) in a completely randomised design.

A corn-soybean meal basal diet was prepared in mash form. Broilers were fed with starter (1–10 d), grower (11–24 d), and finisher (25–42 d) diets formulated according to Ross 308 (Aviagen 2014b) nutrient recommendations except for Met, which was 30% lower (Low-Met) than the recommendation (Table 1). Met level in the basal diet was adjusted at recommendation (Rec-Met) or 30% more than recommendation (High-Met) levels by adding DL- or L-Met (Table 2). Betaine (Sigma Aldrich, St. Louis, MO) was substituted for 30% of supplemental DL- or L-Met according to its methyl donating capacity. Since betaine contains about 3.82 times more methyl groups than Met, supplemental Met was equivalently replaced by betaine (Fu et al. 2016). The photoperiod was 23 L: 1 D (light: dark). Feed and water were provided ad libitum during the whole experimental period. The houses were closed and environmentally controlled. The environmental temperature was 23–25 °C outside the house. In the thermo-neutral control group, ambient temperature was maintained at 32 ± 1 °C on first day. Then, temperature was reduced by 3 degrees per week to reach 27 ± 1 °C at 10 days old and 21 ± 1 °C at 28 days old (Aviagen 2014a). Thereafter, temperature was maintained at 21 ± 1 °C throughout the experiment. Similarly, in the acute heat stress treatment group, ambient temperature was maintained at 32 ± 1 °C on first day. Then, temperature was reduced to 27 ± 1 °C at 10 days old. On day 10, the broilers were subjected to acute heat stress as follows. The ambient temperature (27 °C) was increased over the course of 1.5 h (8:00 a.m. to 9:30 a.m) until 32 °C (60% humidity) using an automated air-forced heater. Subsequently, the temperature was held at 32 °C for 6 h (until 3:30 p.m) and gradually returned to ambient temperature (28 °C) at 5 p.m till the end of the experiment.

Growth performance

The body weight (BW) and feed intake (FI) were recorded periodically on a pen basis, and feed

Table 1. Ingredients and nutrient composition of basal diets, *as-fed basis*[a].

	Starter (0–10 d)	Grower (11–24 d)	Finisher (25–42 d)
Ingredients (%)			
Corn (8% CP)	31.2	25.4	30.0
Soybean meal (44% CP)	39.1	34.0	28.6
Wheat	20.0	30.0	30.0
Soybean oil	5.36	6.74	7.47
Dicalcium phosphate	1.87	1.64	1.70
Limestone	1.14	1.05	1.06
NaCl	0.36	0.35	0.35
DL-or L-Methionine[b]	0.05	0.03	0.03
L-Lysine HCl	0.32	0.27	0.28
L-Threonine	0.1	0.08	0.06
Vitamin premix[c]	0.25	0.25	0.25
Mineral premix[d]	0.25	0.25	0.25
Calculated values (%)			
Metabolisable energy (kcal/kg)	3000 (2993)[e]	3100 (3086)	3200 (3196)
Crude protein	23.0 (23.2)	21.5 (21.2)	19.5 (19.0)
Calcium	0.96 (0.93)	0.87 (0.9)	0.87 (0.86)
Available phosphorus	0.48 (0.45)	0.43 (0.4)	0.43 (0.4)
Sodium	0.16	0.16	0.16
Chloride	0.26	0.24	0.24
Choline (mg/kg)	148.6	140.8	128.6
Total amino acids[f]			
Methionine	0.39 (0.39)	0.36 (0.31)	0.33 (0.30)
Methionine + Cystine	0.76 (0.65)	0.71 (0.60)	0.65 (0.54)
Lysine	1.44 (1.25)	1.29 (1.13)	1.16 (0.99)
Threonine	0.97 (0.78)	0.88 (0.77)	0.78 (0.69)
Isoleucine	0.97 (0.82)	0.89 (0.78)	0.80 (0.67)
Tryptophan	0.35 (0.26)	0.32 (0.25)	0.29 (0.22)
Valine	0.90 (0.79)	0.83 (0.72)	0.75 (0.66)

[a]Met level in the basal diets was 30% lower than recommendation and was increased to recommendation or 30% more than recommendation by supplemental DL- or L-Met; and betaine was or was not substituted for 30% of supplemental DL- or L-Met to provide 12 diets for each rearing phase.
[b]Three other basal diets were also prepared with L-Met.
[c]Vitamin premix provided per kilogram of diet: vitamin A (retinyl acetate), 15,000 U; vitamin D3, 5000 U; vitamin E (DL-α-tocopheryl acetate), 80 mg; vitamin K, 5 mg; thiamine, 3 mg; riboflavin,10 mg; pyridoxine, 5 mg; vitamin B12, 0.02 mg; niacin, 70 mg; choline chloride, 1800 mg; folic acid, 2 mg; biotin, 0.4 mg; pantothenic acid, 20 mg.
[d]Mineral premix provided per kilogram of diet: Mn (manganese sulphate), 100 mg; Zn (zinc sulphate), 65 mg; Cu (copper sulphate), 5 mg; Se (Sodium Selenite), 0.22 mg; I (calcium iodate), 0.5 mg; and Co, 0.5 mg.
[e]The analysed values are presented in parenthesis.
[f]Digestible amino acids are presented in the parenthesis.

Table 2. Analysed vs. calculated methionine and betaine amounts in experimental diets (g/kg).

Treatments	Low-DL or L-Met[a]	Low-DL or L-Met + Bet[b]	Rec[a]- DL or L-Met	Rec- DL or L-Met + Bet[c]	High- DL or L-Met[b]	High- DL or L-Met[b]+Bet[c]
			Starter (1–10 d)			
Supplemented Met	0.50	0.35	2.21	1.54	3.92	2.73
Supplemented betaine	0	0.12	0	0.52	0	0.93
Calculated Met	3.92	3.77	5.63	4.96	7.34	6.15
Calculated cystine	3.64	3.64	3.64	3.64	3.64	3.64
Analysed Met	3.88	3.70	5.59	4.90	7.30	6.10
Analysed cystine	3.60	3.61	3.61	3.59	3.60	3.59
			Grower (11–24 d)			
Supplemented Met	0.30	0.21	1.86	1.31	3.42	2.39
Supplemented betaine	0	0.07	0	0.44	0	0.82
Calculated Met	3.57	3.48	5.13	4.58	6.69	5.66
Calculated cystine	3.52	3.52	3.52	3.52	3.52	3.52
Analysed Met	3.11	3.42	5.08	4.54	6.63	5.60
Analysed cystine	3.47	3.47	3.46	3.45	3.47	3.46
			Finisher (25–42 d)			
Met supplemented	0.30	0.21	1.73	1.22	3.17	2.24
Betaine	0	0.07	0	0.41	0	0.76
Calculated Met	3.29	3.21	4.73	4.22	6.17	5.24
Calculated cystine	3.23	3.23	3.23	3.23	3.23	3.23
Analysed Met	3.04	3.18	4.70	4.19	6.10	5.20
Analysed cystine	3.17	3.17	3.15	3.16	3.17	3.16

[a]Rec: Recommended Met level.
[b]Low-DL or L-Met: methionine level was 30% lower than Ross 308 recommendation; Rec- DL or L-Met: methionine level was as recommended for Ross 308; High-DL or L-Met: methionine level was 30% more than Ross 308 recommendation.
[c]Betaine was substituted for 30% of supplemental DL- or L-Met according to its methyl donating capacity. Betaine contains about 3.82 times more methyl groups compared to Met.

Table 3. Effects of dietary methionine (Met) levels and sources and betaine replacement on performance of broilers grown in normal and heat stress conditions during 11–24 d of age.

Item				Feed intake (g/bird/d)	Weight gain (g/bird/d)	Feed conversion ratio (g:g)	Mortality, %
Temp	Met source	Met level[A]	Betaine[B]				
Heat stress				77.70[b]	52.04[b]	1.512[a]	2.24[a]
Normal				79.63[a]	56.02[a]	1.434[b]	0.68[b]
SEM				0.463	0.361	0.003	0.21
	DL-Met			78.46	53.48	1.480	1.55
	L-Met			78.87	54.57	1.466	1.37
SEM				0.742	0.572	0.018	0.49
		Low-Met		79.75[a]	47.65[b]	1.686[a]	1.83
		Rec-Met[C]		80.09[a]	57.32[a]	1.404[b]	1.27
		High-Met		76.16[b]	57.12[a]	1.339[c]	1.26
SEM				0.913	0.690	0.022	0.60
			+Betaine	78.77	54.48	1.463	1.70
			−Betaine	78.56	53.57	1.487	1.22
SEM				0.748	0.577	0.018	0.49
Source of variation					*p* Value		
Temp				.042	.001	.001	.027
Met source				.702	.181	.399	.790
Met level				.004	<.001	<.001	.753
Betaine				.841	.263	.467	.489
Temp × Met source				.570	.309	.815	.749
Temp × Met level				.843	.107	.207	.503
Temp × Betaine				.924	.764	.630	.749
Met source × Met level				.986	.644	.805	.812
Met level × Betaine				.965	.657	.880	.465
Met source × Betaine				.840	.909	.979	.489

[A]Low-DL or L-Met: methionine level was 30% lower than Ross 308 recommendation; Rec- DL or L-Met: methionine level was as recommended for Ross 308; High-DL or L-Met: methionine level was 30% more than Ross 308 recommendation.
[B]Betaine was substituted for 30% of supplemental DL- or L-Met according to its methyl donating capacity.
[C]Rec: Recommended Met level.
[a-c]Means within a column with no common superscript are significantly different ($p < .05$).

conversion ratio (FCR) was calculated for each period by dividing FI by body weight gain (BWG), taking into account the mortality weights (Imari et al. 2020).

Plasma analysis

On day 42, one bird in each replicate pen was randomly selected that represented the average body weight of the pen. From this, 2.5 mL blood samples were drawn from the wing vein into heparin tubes and were kept on ice to assess the blood uric acid, creatinine and homocysteine concentrations. After centrifugation (3000 xg; 10 min; 4 °C), plasma was collected and stored at −20 °C until further analysis. Plasma samples were analysed for uric acid and creatinine content by a multi-test automatic random access system auto analyser (Cobas Bio, Roche Basel, Switzerland). Plasma hemocycteine was measured by the Axis® Homocysteine EIA kit (Alirezaei et al. 2012).

Hematological profiles

White blood cell differentiation count was assayed on fresh blood samples (via wing vein) on day 42. Individual blood smears were prepared in triplicate glass slides, dried up in the air, and Wright-Giemsa differential was used to stain the slides. One hundred white blood cells were counted under an optic microscope to calculate the heterophil, lymphocyte, eosinophil, basophil, and monocyte, as described by Gross and Siegel (1983).

Muscle collection and histological processing

On day 42, one bird was selected randomly from each pen and then the Pectoralis major of one bird from each replicate was removed from the carcase at, weighed, and then its length and width (mm) were measured by a ruler. The muscle samples (1 cm × 0.5 cm) were immediately fixed in 10% buffered neutral formalin solution for 24 h, dehydrated in alcohol, cleared in xylene, and embedded in paraffin wax. Fibre sample cross-sections were cut at 5 μm thick and stained by haematoxylin and eosin for general tissue morphological evaluation and measuring fibre diameter. Stained cross-sections were captured using a light microscope (Carl ZEISS standard 20, Germany) and a system that analyses computerised images (Dino-lite, Ver. 3.3.0.0, Korea). A total of 100 myofibers per bird were measured in each image by the least diameter method, according to Fernandes et al. (2009).

Table 4. Effects of dietary methionine (Met) level and source and betaine replacement on performance of broilers grown in normal and heat stress conditions during 25–42 d of age.

Item				Feed intake (g/bird/d)	Weight gain (g/bird/d)	Feed conversion ratio (g:g)	Mortality, %
Temp	Met source	Met level[A]	Betaine[B]				
Heat stress				148.95[b]	72.48[b]	2.074[a]	3.24[a]
Normal				154.45[a]	77.22[a]	2.017[b]	1.39[b]
SEM				0.9444	0.378	0.011	0.59
	DL-Met			151.81	74.60	2.060	2.55
	L-Met			151.59	75.10	2.032	2.07
SEM				1.5293	1.051	0.022	0.59
		Low-Met		153.85[a]	68.92[b]	2.242[a]	2.59
		Rec-Met[C]		147.58[b]	76.97[a]	1.931[b]	2.25
		High-Met		153.67[a]	78.65[a]	1.964[b]	2.08
SEM				1.8730	1.287	0.027	0.72
			+Betaine	151.63	75.15	2.035	1.53
			−Betaine	151.77	74.54	2.056	3.09
SEM				1.5293	1.051	0.022	0.59
Source of variation					*p* Value		
Temp				.014	.009	.022	.029
Met source				.922	.735	.381	.561
Met level				.030	<.001	<.001	.882
Betaine				.950	.682	.513	.064
Temp × Met source				.983	.259	.128	.532
Temp × Met level				.968	.624	.556	.718
Temp × Betaine				.974	.867	.817	.898
Met source × Met level				.996	.373	.236	.357
Met level × Betaine				.985	.993	.964	.176
Met source × Betaine				.944	.985	.909	.863

[A]Low-DL or L-Met: methionine level was 30% lower than Ross 308 recommendation; Rec- DL or L-Met: methionine level was as recommended for Ross 308; High-DL or L-Met: methionine level was 30% more than Ross 308 recommendation.
[B]Betaine was substituted for 30% of supplemental DL- or L-Met according to its methyl donating capacity.
[C]Rec: Recommended Met level.
[a–c]Means within a column with no common superscript are significantly different ($p < .05$).

Statistical analysis

Each response parameter was analysed as a $2 \times 2 \times 3 \times 2$ split-plot factorial arrangement with temperature as main-plot and diet as sub-plot. Pen means were the experimental units for all statistical analyses. Statistical analysis was performed using the GLM procedures of SAS software (SAS Institute 2012), and differences between treatment means were specified with Turkey's test. All statements of significance were based on $p < .05$.

Results

Growth performance

The effects of dietary treatments on the growth performance of the birds reared under normal and heat stress conditions during grower (11–24 d) and finisher (25–42 d) periods are shown in Tables 3 and 4. Feed intake and FCR were the lowest in High-Met treatment, while BWG was higher in Rec- and High-Met treatments compared to the Low-Met treatments during 11–24 days of age ($p < .05$). Body weight gain and FCR were higher and lower in both Rec- and High-Met treatments compared to the Low-Met treatments, respectively, during 25–42 days of age ($p < .05$). Feed intake, BWG, and FCR were not significantly influenced by Met source and betaine replacement. The cyclic HS had negative effects on FI, BWG, and FCR and significantly increased mortality during 11 to 24 and 25 to 42 d of age ($p < .05$). There were no significant interaction effects among the experimental groups ($p > .05$).

Plasma metabolites

The effects of Met levels and sources and betaine replacement for supplemental Met on plasma uric acid, creatinine, and homocysteine concentrations of heat-stressed birds are shown in Table 5. Plasma uric acid concentration was not affected by the Met type ($p > .05$). Replacing 30% of the supplemental Met with betaine showed a similar result on uric acid concentration compared to non-supplemented betaine diets ($p > .05$). There was an interaction effect between Met levels and temperature for plasma uric acid concentration ($p = .036$); so that the heat-stressed birds fed with the highest level of Met had higher uric acid concentration than those fed with the other levels of Met under normal and stress conditions (Table 6).

Met levels influenced plasma creatinine concentration, and the highest level of Met significantly increased creatinine level compared to other Met

Table 5. Effects of dietary methionine (Met) levels and sources and betaine replacement on plasma uric acid, creatinine, and homocysteine concentration of broilers grown in normal or heat stress conditions at 42 d of age.

Treatments				Uric acid, mg/dL	Creatinine, mg/dL	Homocysteine, μmol/L
Temp	Met source	Met Level[A]	Betaine[B]			
Heat stress				6.11[a]	0.454	23.89[a]
Normal				5.62[b]	0.471	21.87[b]
SEM				0.099	0.011	0.248
	DL-Met			5.87	0.444	22.88
	L-Met			8.80	0.461	22.93
SEM				0.111	0.014	0.331
		Low-Met		5.09[b]	0.384[b]	16.38[c]
		Rec-Met[C]		5.50[b]	0.395[b]	19.11[b]
		High-Met		7.01[a]	0.579[a]	33.14[a]
SEM				0.136	0.017	0.406
			+Betaine	5.87	0.446	22.26[b]
			−Betaine	5.86	0.460	23.49[a]
SEM				0.111	0.014	0.331
Source of variation						
					p Value	
Temp				.025	.086	.010
Met source				.999	.384	.836
Met level				.001	.001	>.001
Betaine				.927	.491	.010
Temp × Met source				.194	.534	.172
Temp × Met level				.036	.304	.006
Temp × Betaine				.520	.097	.195
Met source × Met level				.978	.178	.561
Met level × Betaine				.584	.791	.405
Met source × Betaine				.389	.431	.737

[A]Low-DL or L-Met: methionine level was 30% lower than Ross 308 recommendation; Rec- DL or L-Met: methionine level was as recommended for Ross 308; High-DL or L-Met: methionine level was 30% more than Ross 308 recommendation.
[B]Betaine was substituted for 30% of supplemental DL- or L-Met according to its methyl donating capacity.
[C]Rec: Recommended Met level.
[a-c]Means within a column with no common superscript are significantly different ($p < .05$).

Table 6. The significant interaction of methionine (Met) levels[A] with temperature on plasma uric acid and homocysteine concentration of broilers grown in normal and heat stress conditions.

Item		Uric acid, mg/dL	Homocysteine, μmol/L
Heat stress	Low-Met	5.11[c]	17.71[c]
	Rec-Met[B]	5.69[c]	21.10[b]
	High-Met	7.53[a]	32.85[a]
Normal	Low-Met	5.06[c]	15.05[d]
	Rec-Met	5.31[c]	17.11 [cd]
	High-Met	6.50[b]	33.44[a]
SEM		0.192	0.574
p Value		.036	.006

[A]Low-DL or L-Met: methionine level was 30% lower than Ross 308 recommendation; Rec- DL or L-Met: methionine level was as recommended for Ross 308; High-DL or L-Met: methionine level was 30% more than Ross 308 recommendation.
[B]Rec: Recommended Met level.
[a-d]Means within a column with no common superscript are significantly different ($p < .05$).

levels ($p < .05$). Met type, betaine replacement, and HS did not affect the creatinine concentration of plasma ($p > .05$).

Plasma homocysteine was not influenced by the Met type ($p > .05$). Replacing 30% of the supplemental Met with betaine showed lower homocysteine concentration compared to non-supplemented betaine diets fed birds ($p = .01$). A significant interaction between the Met level and temperature showed that birds fed Low- and Rec-Met diet under normal condition had lower homocysteine concentration than their counterparts under HS condition (Table 6).

Hematological profile

White blood cells differential count is shown in Table 7. The Met source had no significant effect on white blood cells count ($p > .05$). Betaine replacement for 30% of supplemental Met resulted in similar consequences compared to non-Bet replacement diets on white blood cells count ($p > .05$). Percentages of heterophil, lymphocyte, and H/L ratio were affected by the interaction of Met level and temperature ($p < .05$) that are shown in Table 8. The High-Met reduced the H:L ratio under heat stress but not in thermoneutral conditions.

Breast muscle characteristics and histological traits

Results concerning analyses of variance on muscle yield, length, width, and myofiber diameter of pectoralis muscle are shown in Table 9. The lowest level of Met showed lower muscle yield and width than the other two levels of Met ($p < .0001$). Betaine replacement for 30% of supplemental Met resulted in similar

Table 7. Effects of dietary methionine (Met) levels and sources and betaine replacement on white blood cell differential count in broilers grown in normal or heat stress conditions at 42 d of age.

Treatments									
Temp	Met source	MetLevel[A]	Betaine[B]	Heterophil, %	Lymphocyte, %	H/L ratio	Monocyte, %	Eosinophil, %	Basophil, %
Heat stress				38.95[a]	54.03[b]	0.72[a]	3.25	2.35	1.41
Normal				36.08[b]	57.11[a]	0.63[b]	3.15	2.36	1.43
SEM				0.137	0.237	0.006	0.075	0.077	0.086
	DL-Met			37.43	55.50	0.68	3.16	2.43	1.46
	L-Met			37.60	55.65	0.68	3.23	2.28	1.38
SEM				0.433	0.438	0.012	0.07	0.076	0.067
		Low-Met		39.27[a]	53.87[b]	0.73[a]	3.17	2.50	1.40
		Rec-Met[C]		37.67[a]	55.25[b]	0.68[a]	3.12	2.35	1.42
		High-Met		35.60[b]	57.60[a]	0.62[b]	3.30	2.22	1.45
SEM				0.531	0.537	0.015	0.083	0.093	0.082
		+Betaine		37.50	55.68	0.68	3.21	2.31	1.43
		−Betaine		37.53	55.46	0.68	3.18	2.40	1.41
SEM				0.433	0.438	0.012	0.068	0.076	0.067
Source of variation				*p* Value					
Temp				<.001	.0008	.0005	.401	.886	.897
Met source				.786	.809	.926	.493	.167	.384
Met level				<.001	<.001	<.001	.320	.119	.912
Betaine				.956	.727	.796	.731	.441	.861
Temp × Met source				.745	.893	.839	.305	.643	.861
Temp × Met level				.007	.002	.003	.915	.733	.468
Temp × Betaine				.828	.768	.740	.172	.440	.861
Met source × Met level				.910	.728	.888	.572	.171	.561
Met level × Betaine				.852	.698	.817	.970	.733	.673
Met source × Betaine				.626	.323	.428	.305	.167	.384

[A]Low-DL or L-Met: methionine level was 30% lower than Ross 308 recommendation; Rec- DL or L-Met: methionine level was as recommended for Ross 308; High-DL or L-Met: methionine level was 30% more than Ross 308 recommendation.
[B]Betaine was substituted for 30% of supplemental DL- or L-Met according to its methyl donating capacity.
[C]Rec: Recommended Met level.
[a-b]Means within a column with no common superscript are significantly different ($p < .05$).

Table 8. The significant interaction of methionine (Met) levels[A] with temperature on heterophil, lymphocyte and H/L ratio in broilers grown in normal and heat stress conditions.

Item		Heterophil, %	Lymphocyte, %	H/L ratio
Heat stress	Low-Met	41.60[a]	51.25[b]	0.81[a]
	Rec-Met[B]	39.60[ab]	53.25[b]	0.74[ab]
	High-Met	35.65[c]	57.6[a]	0.62[c]
Normal	Low-Met	36.95[bc]	56.50[a]	0.66[bc]
	Rec-Met	35.75[c]	57.25[a]	0.63[c]
	High-Met	35.55[c]	57.60[a]	0.61[c]
SEM		0.751	0.760	0.022
p Value		.007	.002	.003

[A]Low-DL or L-Met: methionine level was 30% lower than Ross 308 recommendation; Rec- DL or L-Met: methionine level was as recommended for Ross 308; High-DL or L-Met: methionine level was 30% more than Ross 308 recommendation.
[B]Rec: Recommended Met level.
[a-c]Means within a column with no common superscript are significantly different ($p < .05$).

consequences comparing to non-betaine replacement diets on the muscle yield, length, width, and myofiber diameter of pectoralis muscle ($p > .05$). The significant interaction was observed between the Met source and temperature for greater breast width ($p = .041$); so that the birds fed with the L-Met diet had more breast width under thermal stress than birds fed with DL-Met (Table 10); but there was no significant difference between birds fed with DL or L-Met under the normal temperature condition ($p > .05$). The interactions between Met level and temperature, as well as Met level and Met source, were significant for the myofibril diameter in the pectoralis muscle (Table 10). High-Met diet had a higher myofiber diameter under normal temperature conditions than Low- and Rec-Met diet under thermal stress condition ($p = .036$). Birds fed diets containing the highest L-Met levels had a greater myofibril diameter than those fed the other three levels of DL-Met ($p = .012$). The significant interaction was observed between the Met level and temperature for breast yield ($p = .031$). High-Met diet had a higher muscle yield under normal temperature conditions compared to their counterparts under HS (Table 10).

Discussion

Growth performance

From 11 to 24 d of age, the best FCR response was observed for High-Met group, but no difference in BWG was observed between Rec- and High Met. It is similar to the results of Wen et al. (2014), who reported broilers fed High Met diets had a greater

Table 9. Effects of dietary methionine (Met) levels and sources and betaine replacement on breast yield, breast length, breast width, and pectoralis major myofibers diameter in broilers grown in normal or heat stress conditions at 42 d of age.

Treatments				Breast yield, %	Breast length, mm	Breast width, mm	Myofiber diameter, μm
Temp	Met source	Met Level[A]	Betaine[B]				
Heat stress				26.77[b]	148.80	118.27[b]	21.90[b]
Normal				28.02[a]	149.84	120.80[a]	23.28[a]
SEM				0.164	0.444	0.612	0.351
	DL-Met			27.23	149.49	118.47[b]	21.33[b]
	L-Met			27.56	149.15	120.60[a]	22.85[a]
SEM				0.282	0.668	0.610	0.386
		Low-Met		25.99[b]	148.32	114.5[b]	18.17[b]
		Rec-Met[C]		27.90[a]	149.96	122.9[a]	23.62[a]
		High-Met		28.30[a]	149.67	121.1[a]	24.47[a]
SEM				0.346	0.819	0.748	0.476
			+Betaine	27.47	149.81	119.5	22.2
			-Betaine	27.32	148.82	119.4	21.9
SEM				0.282	0.668	0.610	0.388
Source of variation				*p* Value			
Temp				.005	.172	.043	.008
Met source				.421	.718	.015	.007
Met level				<.001	.324	>.001	>.001
Betaine				.709	.297	.992	.607
Temp × Met source				.846	.568	.041	.927
Temp × Met level				.031	.752	.761	.036
Temp × Betaine				.846	.667	.483	.975
Met source × Met level				.928	.482	.299	.012
Met level × Betaine				.422	.658	.790	.516
Met source × Betaine				.470	.414	.946	.175

[A]Low-DL or L-Met: methionine level was 30% lower than Ross 308 recommendation; Rec- DL or L-Met: methionine level was as recommended for Ross 308; High-DL or L-Met: methionine level was 30% more than Ross 308 recommendation.
[B]Betaine was substituted for 30% of supplemental DL- or L-Met according to its methyl donating capacity.
[C]Rec: Recommended Met level.
[a-b]Means within a column with no common superscript are significantly different ($p < .05$).

Table 10. The significant interaction of temperature, methionine (Met) levels[A] and source on breast yield, width and myofiber diameter in broilers grown in normal and heat stress conditions.

Item		Breast yield	Breast width, mm	Myofiber diameter, μm
Heat stress	Low-Met	25.95[c]		18.00[d]
	Rec-Met[B]	27.40[bc]		21.85[c]
	High-Met	26.97[bc]		22.85[bc]
Normal	Low-Met	26.02[c]		18.35[d]
	Rec-Met	28.41[ab]		25.40[ab]
	High-Met	29.63[a]		26.10[a]
SEM		0.490		0.673
p Value		0.031		.036
Heat stress	DL-Met		116.31[b]	
	L-Met		120.23[a]	
Normal	DL-Met		120.63[a]	
	L-Met		120.96[a]	
SEM			0.863	
p Value			.041	
DL-Met	Low-Met			18.30[c]
	Rec-Met			23.10[b]
	High-Met			22.60[b]
L-Met	Low-Met			18.05[c]
	Rec-Met			24.15[ab]
	High-Met			26.35[a]
SEM				0.673
p Value				.012

[A]Low-DL or L-Met: methionine level was 30% lower than Ross 308 recommendation; Rec- DL or L-Met: methionine level was as recommended for Ross 308; High-DL or L-Met: methionine level was 30% more than Ross 308 recommendation.
[B]Rec: Recommended Met level.
[a-c]Means without common superscript are significantly different ($p < .05$).

($p < .05$) G:F than the control birds throughout the experiment, but no difference in BWG was observed.

Also, findings of growth performance confirmed the reports of earlier researchers, who reported that BWG was significantly higher by 110% and 130% of NRC methionine than that of the control diet (Rehman et al. 2019). Although, Whitaker et al. (2002) concluded that broilers' performance was not affected by dietary Met levels from 100 to 140% of the recommendation. On the other hand, it is rather intuitive that a 30% reduction of dietary Met levels (Low-Met) would have affected productive performance.

Replacement of 30% of supplemental Met with betaine, in our study, did not affect broilers' performance, which implied that betaine might have a sparing effect for methionine. Betaine-Homocysteine-Methyltransferase (BHMT) facilitates the transfer of methyl groups from betaine to homocysteine. This process is irreversibly converted to cysteine for protein synthesis, or re-methylated by other methyl sources to Met. Other authors have noted that a portion of the Met requirement could be covered by betaine supplementation in diets marginally deficient in Met, while attempts to replace too much of the Met requirement with betaine have been unsuccessful (Pillai et al.

2006). Several factors may affect the variability in response, including age and sex of broilers, dietary factors, and management (Eklund et al. 2005; Zhan et al. 2006).

The main effect of the Met source on the growth performance of the chicks was not significant. Some authors reported the same efficacy between L-Met and DL-Met (Ribeiro et al. 2005; Dilger and Baker 2007). But there are reports that show L-Met has a higher efficiency than DL-Met (Shen et al. 2014, 2015; Park et al. 2018). Shen et al. (2015) showed that the relative bioavailability of L-Met is higher than DL-Met in broilers.

It is well known that heat stress imposes several sever changes on birds' physiological functions, such as reducing feed intake, intestinal dysfunction, hormone secretion, and electrolyte imbalance, leading to impaired production function (Quinteiro-Filho et al. 2010; Lu et al. 2017). The results of the present study are in line with those reported by Cengiz et al. (2015) and Hosseini et al. (2016), who indicated an impairment of growth performance during HS condition.

Chickens under heat stress expend more energy, adapting to high ambient temperatures. As a result, growth performance is impaired (Nawab et al. 2018). In addition, part of the negative effects of HS may be due to the increased production of reactive oxygen species (ROS), which have adverse effects on the constituents of biological tissues (protein, amino acids, lipids, and DNA), leads to poor performance in broiler chickens. Also, modification of hypothalamic peptides involved in appetite regulation, a decrease passage rate of feed residue, changes in intestinal morphology, and nutrient absorption are other deleterious effects of HS (Song et al. 2014; Attia and Hassan 2017).

Plasma metabolites

Serum uric acid concentration can be used as an indicator of amino acid utilisation in broilers (Donsbough et al. 2010). In our study, uric acid concentration was affected by Met levels, which was consistent with those of Wen et al. (2014), who reported that with increasing total sulphur amino acid content in Met supplemented diet, nitrogen catabolism increases uric acid production. In line with our results Azad et al. (2010) reported that plasma uric acid level tended to increase with constant 32HS and was increased ($p < .05$) with 34HS. Lin et al. (2006) and Willemsen et al. (2011) reported that the plasma concentration of uric acid was not significantly changed by acute heat exposure. This contradiction may relate to the less severe extent of stress. The duration of heat stress seems to influence differently the protein metabolism of animals (Belhadj Slimen et al. 2016).

A 30% substitution of supplemental Met with betaine resulted in similar uric acid concentration in the present study. Zhan et al. (2006) reported that betaine supplementation in a diet with Met deficiency decreased serum uric acid concentration in 22-day-old broilers, which is not consistent with our results. Some possible reasons for this inconsistent result may be the differences in the basal diets, different environmental conditions, and the bird's age. In our study, plasma creatinine levels increased with increasing Met levels from deficient (Low-Met) to 30% higher (High-Met). Hasegawa et al. (2017) declared that Met or arginine might become a limiting amino acid for creatine synthesis when Met or arginine was deficient in the diet. Other creatine precursors might become limiting amino acids for creatine biosynthesis when Met or arginine was excess in diet. The results of Del Vesco et al. (2014) indicated that the interaction between diet and environment influenced uric acid concentration, and the highest concentration of uric acid was observed in birds fed with Met supplemented diet under thermal stress condition.

In this study, the lower concentration of homocysteine in birds raised under normal temperature conditions is probably due to the normal metabolism and thus high expression of the BHMT enzyme. Del Vesco et al. (2015) revealed that the gene expression of BHMT was lower when the heat-stressed birds were fed the Met deficient diet, thereby indicating that the organism under stress can stimulate the production of glutathione even when fed low Met diets. Given the important role of Met in glutathione synthesis, the most of metabolically available homocysteine under stress conditions is directed towards glutathione synthesis (Persa et al. 2004). In the present study, the highest concentration of homocysteine was observed in birds fed with the highest level of Met under normal and stress conditions. This may be due to sufficient Met supply and lower BHMT expression, which leads to increased plasma homocysteine concentrations (Del Vesco et al. 2015). The Met type did not affect the plasma levels of homocysteine, as shown in Table 5. Betaine replacement reduced the level of homocysteine in plasma. Catabolism of betaine involves a series of reactions that result in the transmethylation of homocysteine to Met, resulting in the production of di-methylglycine and a decrease in the plasma concentration of homocysteine (Williams and Schalinske 2007). Under Met-deficient conditions, a

large increase in BHMT activity may occur, especially in the presence of excess choline or betaine (Emmert et al. 1996). This can accelerate the conversion of homocysteine to Met and mitigate Met deficiency.

Hematological profiles

Under stressful environmental conditions, as the bird's body attempts to maintain its thermal homeostasis, ROS production increases. Consequently, the body enters the oxidative stress state and begins to produce and release heat shock proteins to protect itself against the deleterious cellular effects of ROS (Lara and Rostagno 2013). Therefore, the decrease in H/L ratio in heat-stressed birds fed a diet containing 130% Recommended Met level may be due to the fact that the increased need for amino acids in these conditions leads to the synthesis of proteins or other specific compounds such as hormones and heat shock proteins that alleviate the adverse effects of HS. Shini et al. (2005) revealed that Met requirements for optimal immunity is greater than that of growth, and lower sulphur amino acids such as Met and cysteine, leading to a severe lymphocyte depletion into the intestine tissues (Swain and Johri 2000).

It is proven that the H/L ratio is an indicator of the hypothalamoadeno-pituitary-adrenal (HPA) axis activity and a stress indicator in poultry (Virden and Kidd 2009). Recent studies have shown that HS affects white blood cells and increases heterophil percentage and H/L ratio through glucocorticoid secretion (Prieto and Campo 2010; Quinteiro-Filho et al. 2010). In the current study, HS condition increased the H/L ratio regardless of the Met level, consistent with the previous studies (Yalcin et al. 2003; Akşit et al. 2006; Zulkifli et al. 2009). The H/L ratio was 0.72 for the heat-stressed birds. This indicates a high effect of HS, bearing in mind that 0.2, 0.5, and 0.8 for the H/L ratio are characteristic of low, optimal, and high degrees of stress, respectively (Prieto and Campo 2010).

Pectoral muscle histology

This study demonstrated that breast yield was improved by increasing Met levels from deficient to the recommendation and 30% higher than the recommendation. This result is consistent with data of Zhai et al. (2012), who reported that breast meat yield in birds fed diets containing 0.51% Met was higher in 42 days of age than birds fed diets containing 0.41% Met from 22 to 42 days of age. So, Met promotes broiler growth by regulating the development of breast muscle. This may be due to that Met was increased muscle protein deposition (Nagao et al. 2011; Zhai et al. 2012). Muscle yield was decreased under HS condition than normal temperature condition in Rec- and High-Met diet. Zhang et al. (2012) reported that heat stress reduced breast muscle yield in broilers. Sahin and Seyrani (2014) reported that increasing levels of methionine (0.025 and 0.05%) significantly reduced carcase weight and breast muscle weight in two different temperature conditions (30 and 21 °C). Carcase weight loss may be due to insufficient intake of energy and nutrients and reduced production and storage of glycogen as the most important source of energy (Geraert et al. 1996).

Basic fibroblast growth factor-2 (FGF-2) is a factor that stimulates proliferation and inhibits the differentiation of muscle cells (Velleman 2007). Interactions between muscle cells and FGF-2 depend on the presence of heparan sulphate in the extracellular matrix. FGF-2 exhibits characteristic complex formation with the heparan sulphate and vascular endothelial receptors, and loss of sulphur groups blocks its activity (Casu et al. 2004). Zielinska et al. (2012) reported that after the administration of taurine, which has a sulphur group and can be a donor of sulphur compounds, the structure of the connective tissue holding the fiber bundles was strong, the fibers were homogeneous and mature, and large spaces between the bundles of fibers were observed. In the present study, broilers fed a diet with 130% of Recommended Met level under normal conditions had a higher fibre diameter than the other two groups under stress condition. A probable explanation for this result could be a shortage of sulphur groups in the diets containing Met at the requirement or less than the requirement, suggesting that Met, as a donor of sulphur group, enhanced muscle fibre diameter.

We observed an interaction between Met isomers and thermal conditions on breast width and an interaction between Met isomers and Met level on myofibril diameter, although the mechanism underlying this observation is unclear. In animals, L- and DL-methionine isomers follow the sodium-dependent and sodium-independent pathways to traverse the gut wall (Knight et al. 1994; Soriano-García et al. 1998). During heat stress, absorption via the sodium-independent pathway results in lower uptake of D-Met than that of L- Met. Consequently, conversion of D-Met to L- Met leads to energy loss under HS condition (Knight et al. 1994). Therefore, L- Met has a greater advantage in broilers during heat stress. However, Yang et al. (2019) indicated that there was not

difference in the expression of myogenesis-related genes and muscle growth in pigs fed with L-Met and DL-Met.

In the present study, the substitution of 30% of supplemental Met with betaine resulted in similar myofiber diameter comparing to non-substituted diets. No focussed studies have been done on the cellular and molecular mechanisms of betaine on skeletal muscle differentiation and hypertrophy of the chest muscle. The results of Senesi et al. (2013) showed that betaine is a positive stimulator of the IGF-1 pathway in the chicken breast muscle. During betaine treatment, animals have shown an increase in growth hormone, IGF-1, and insulin in addition to an increase in muscle mass, indicating the association between betaine's action on muscle and signalling of IGF-1 (Senesi et al. 2013).

Conclusions

The best FCR response was observed for High-Met group from 11 to 24 d of age, but no difference in BWG was observed between Rec- and High Met. An increase of the dietary Met supply to 130% of the recommendations caused enhanced plasma metabolites and decreased H/L ratio. The use of L-Met in the diet was more effective in increasing myofiber diameter compared with DL-Met supplementation. In addition, we observed that betaine functions were similar to that of Met, so it seems that 30% of the supplemental Met can be replaced with betaine. Heat stress impeded growth performance, muscle yield and development, and immune response of broilers, irrespective of another variable.

Ethical approval

All procedures were approved by the Animal Care and Use Committee of the Ferdowsi University of Mashhad, Mashhad, Iran.

Disclosure statement

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