Received: 29 December 2017 | Revised: 29 July 2018 | Accepted: 13 September 2018

DOI: 10.1111/jpn.13005

ORIGINAL ARTICLE

WILEY Journal of Animal Physiology and Animal Nutrition

Effects of dietary supplemental methionine source and betaine replacement on the growth performance and activity of mitochondrial respiratory chain enzymes in normal and heat-stressed broiler chickens

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Funding information
Ferdowsi University of Mashhad, Grant/Award Number: 3/38938

Abstract

This study aimed to evaluate the effects of dietary supplemental methionine (Met) source and betaine (Bet) replacement for Met on performance and activity of mitochondrial respiratory chain enzymes (MRCEs) in normal and heat-stressed broiler chickens. Total of 1,200-day-old Ross 308 chicks were allocated to two houses, each consisted of 12 treatments, five replicates of 10 birds each with 2 × 2×3 × 2 (temperature × Met source × Met level × Bet, respectively) split-plot factorial arrangement. Met level in the basal diets was 70% requirements (Req) that was increased to the requirement or 130% by supplemental dl- or l-Met. Bet was or was not substituted at the rate of 30% supplemental dl- or l-Met. Feed conversion ratio (FCR) in chicks fed 70% l-Met was lower than those fed 70% dl-Met diet during 1–10 days ($p = 0.04$). Broilers fed diets containing requirement or 130% Met, regardless of its source, showed higher weight gain (WG) than those received 70% Met diet during 11–42 days ($p < 0.001$). Feed intake (FI) of broilers fed 130% Met diet was decreased compared to other two groups during 11–42 days ($p < 0.05$). One hundred thirty percent Met requirement diet resulted in lower FCR comparing to other two groups during 11–42 days ($p < 0.001$). Heat-stressed birds grew less than those under normal condition ($p < 0.05$). Broilers fed Req Met diet under normal temperature exhibited higher activities of complexes (Cox) I and III ($p < 0.05$). Cox I activity in heat-stressed birds fed Bet + diet was similar to those fed Bet-diet under normal temperature ($p = 0.046$). It is concluded that performance and the activities of Cox I and III were increased as the level of Met increased. Bet replacement for 30% supplemental Met resulted in similar consequences comparing to non-Bet replacement diets on performance, but increased the activity of Cox III. l-Met was effective than dl-Met at the cellular level. High ambient temperature depressed performance and MRCE activity.

KEYWORDS
betaine, broiler, heat stress, methionine, mitochondria

1 | INTRODUCTION

Heat stress is considered as one of the most important stressors which is accompanied by economic losses in the poultry industry worldwide; particularly in the hotter regions of the world (Tan, Yang, Fu, Feng, & Zhang, 2010). It has deleterious effects on broiler productivity and welfare and alleviates feed intake, body weight gain. Heat stress may increase feed:gain ratio by altering biological molecules, disturbing cell functions and metabolic reactions, and inducing oxidative cell damage (Du, Di, Guo, Li, & Wang, 2008; Pandey et al., 2012). Therefore, heat stress induces oxidative injury in which reactive oxygen species (ROS) production is in excess of the antioxidant capacity of animal cells (Halliwell & Whiteman, 2004). When the steady state of ROS production is disturbed, mitochondria are the first cellular compartment that is damaged (Belhadj Slimen, Najar, Ghram, & Abdrrabba, 2016). Bottje et al. (2002) stated that mitochondria are responsible for producing 90% cell energy, so some of the variations in broiler growth performance (Emmerson, 1997) may be related to the differences or inefficiencies in mitochondrial function. Also, relationship between feed efficiency, growth and mitochondrial function in poultry, rats, and cattle have been reported in numerous studies (Bottje & Carstens, 2009; Goolish & Adelman, 1987; Iqbal et al., 2004; Pelletier, Dutil, Blier, & Guderley, 1994; Toyomizu, Kirihara, Tanaka, Hayashi, & Tomita, 1992).

Methionine has a substantial role in maintaining glutathione (GSH) concentrations that known as one of the most important antioxidants in both mammalian and avian species (Willemsen et al., 2011). In addition, Met as a central player in heat stress resistance is a direct target of ROS. Methionine can act as a scavenger which protects cells from oxidative stress by oxidation of its sulphur to sulfoxide and repairing Met sulfoxide through Met sulfoxide reductases (Campbell, Vowinckel, Keller, & Ralser, 2016). Therefore, additional dietary Met supplement may contribute to the antioxidant status of broiler chickens.

In poultry diets, Met can be fed as a racemic mixture, but only l-Met can be directly used for protein synthesis. The key enzyme that converts isomer d to l-Met is expressed mostly in kidney, liver and intestinal cells. d-Met should be first converted to l-Met until can be utilized directly by the cells. Therefore, l-Met is the only biological form of Met that is readily utilized by the gastrointestinal tract (GIT) cells (Shen, Ferket, Park, Malheiros, & Kim, 2015). From this point, Met has various metabolic fates in many important physiological processes (Metayer et al., 2008).

The physiologic role of Bet is either as an organic osmolyte to protect cells under heat stress or as a catabolic source of methyl groups in transmethylation reactions that involve the transfer of methyl groups to homocysteine. Bet can re-methylate homocysteine to Met by mediating betaine–homocysteine methyltransferase (BHMT) in the Met cycle (Schafer et al., 2007). Also, studies showed the protective effect of Bet on mitochondrial function (Ganesan, Rajesh, Anandan, & Dhandapani, 2007). Therefore, the present study aimed to evaluate the effects of supplemental Met sources (dl

TABLE 1 Ingredients and nutrient composition of basal diets (g/kg as-fed basis)[a]

	Starter (0–10 days)	Grower (11–24 days)	Finisher (25–42 days)
Ingredients			
Corn, 8 g CP/kg	312	254	300
Soybean meal, 44 g CP/kg	391	340	286
Wheat	200	300	300
Soybean oil	53.6	67.4	74.7
Dicalcium phosphate	18.7	16.4	17
Limestone	11.4	10.5	10.6
NaCl	3.6	3.5	3.5
dl-Methionine[b]	0.5	0.3	0.3
l-Lysine HCl	3.2	2.7	2.8
l-Threonine	1.0	0.8	0.6
Vitamin premix[c]	2.5	2.5	2.5
Mineral premix[d]	2.5	2.5	2.5
Calculated values (g/kg)[e]			
Metabolizable energy (MJ/kg)	12.56 (12.51)[f]	12.98 (12.90)	13.40 (13.36)
Crude protein	230 (232)	215 (212)	195 (190)
Calcium	9.6 (9.3)	8.7 (9.0)	8.7 (8.6)
Available phosphorus	4.8 (4.5)	4.3 (4.0)	4.3 (4.0)
Sodium	1.6	1.6	1.6
Chloride	2.5	2.4	2.4
Choline	1.73	1.66	1.53
Total amino acids[g]			
Methionine	3.92 (3.88)	3.57 (3.11)	3.29 (3.04)
Methionine + cystine	7.56 (6.46)	7.09 (5.98)	6.52 (5.42)
Lysine	14.4 (12.5)	12.9 (11.3)	11.6 (9.87)
Threonine	9.70 (7.80)	8.80 (7.74)	7.80 (6.94)
Isoleucine	9.69 (8.23)	8.91 (7.75)	8.02 (6.73)
Tryptophan	3.49 (2.63)	3.22 (2.48)	2.86 (2.23)
Valine	8.99 (7.91)	8.25 (7.17)	7.45 (6.55)

[a]Met level in the basal diets (70% methionine requirement) increased to requirement or 130% of requirement, 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 IU; vitamin D3, 5,000 IU; vitamin E (dl-α-tocopheryl acetate), 80 mg; vitamin K, 5 mg; thiamin, 3 mg; riboflavin, 10 mg; pyridoxine, 5 mg; vitamin B12, 0.02 mg; niacin, 70 mg; choline chloride, 350 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 values were calculated from Aviagen (2014). [f]The analysed values are represented in parenthesis. [g]Digestible amino acids are represented in the parenthesis.

TABLE 2 Analysed versus calculated methionine and betaine level in all diets (g/kg)

Treatment	70%Met[a]	70% Met[a] + betaine[b]	Req Met	Req Met + betaine[b]	130% Met[a]	130% Met[a] + betaine[b]
Starter (1–10 days)						
Met, supplemented	0.50	0.35	2.21	1.54	3.92	2.73
Betaine, supplemented	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 days)						
Met, supplemented	0.30	0.21	1.86	1.31	3.42	2.39
Betaine, supplemented	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 days)						
Met, supplemented	0.30	0.21	1.73	1.22	3.17	2.24
Betaine, supplemented	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

Notes. Req: requirement Met.
[a]70% Met, methionine level was 30% lower than Ross 308 recommendation; 130% 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. Betaine contains about 3.82 times more methyl groups compared to Met.

or l) and Bet replacement for Met on the growth performance and MRCE activities in normal and heat-stressed broiler chickens.

2 | MATERIALS AND METHODS

2.1 | Experimental design, diets and housing

This experiment was carried out in two attached poultry houses (n = 1,200, Ross 308) with similar conditions except for extra heating system for heat stress induction. All procedures used were approved by the Ferdowsi University of Mashhad Animal Care and Use Committee. The experiment was designed in a 2 × 2×3 × 2 (temperature, Met source, Met level and Bet replacement respectively) split-plot factorial arrangement in two poultry houses each containing 60 pens (1.2 m × 1 m) as the main plot and 12 diets as the subplot, with five replicates of 10 birds in each pen (initial body weights; 42 ± 1.2 g). Mash corn–soybean meal basal diets were prepared for days 1–10 (starter) and 11–42 to meet 2014 Ross 308 nutrient recommendations, except for Met (Aviagen, 2014; Table 1).

Met level in the basal diets was adjusted at 70% requirements. Then, the Met level was increased to requirement or 130% requirement by dl- or l-Met supplementation (Table 2). Bet was or was not substituted with 30% of supplemental dl-Met or l-Met according to its methyl-donating property. Bet contains about 3.82 times methyl groups compared with Met (Fu et al., 2016). The lighting program was 23L: 1D, and feed and water were provided ad libitum throughout the whole experimental period. The temperature of both houses was set at thermal comfort during 1–10 days of age. Then, one of houses temperature was gradually decreased by approximately 3°C/week until it reached 22°C temperature at day 28 and remained constant thereafter. In the other house, in order to apply thermal stress, daily temperature was gradually increased to 32°C (08:00 and 09:30) and was maintained for 6 hr (15:30) and then was gradually decreased to the recommended temperature (17:00) from 11 to 42 days of age. Average relative humidity was kept at 40% during rearing period using a water spray device.

2.2 | Growth performance and sample collection

All birds were weighed at the beginning and at the end of each rearing period. FI was calculated as the difference between the amount of feed offered and the feed residue at the end each period. FCR was calculated by dividing FI by WG and corrected for mortality.

2.3 | Isolation of mitochondria

Isolation of muscle mitochondria from pectoralis superficialis tissue was performed by differential centrifugation as described by Kolath, Kerley, Golden, and Keister (2006). At the end of rearing period (42 days of age), five birds per treatment were selected randomly and killed by cervical dislocation. Then, about 150 mg of breast muscle per bird was taken in 1 ml of ice cold isolation medium I (100 mM sucrose, 100 mM Tris-HCl, 46 mM KCl, 10 mM EDTA, pH 7.4). The tissue was chopped using sharp a scissor and incubated in medium I, containing 5 mg of protease K for 5 min at room temperature. After gentle homogenization for 90 s using a Teflon homogenizer, it was stood for 5 min on ice, and the homogenate was centrifuged (1,000 *g* for 10 min at 4°C) then, resulting supernatant was separated and centrifuged (10,000 *g* for 15 min at 4°C) and was eliminated. The resulting pellet was washed in medium I plus 0.5% BSA and centrifuged again (10,000 *g* for 15 min at 4°C). Then, the final mitochondrial pellet was suspended in 200 µl of medium II (230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 5 mM KH_2PO_4, pH = 7.4) and stored at −80°C until further analysis. Bradford method (Lawrence & Davies, 1986) was used to determine the protein concentrations of the mitochondrial extracts (isolated as described above) using spectrophotometer (UV) at 595 nm wavelength.

2.4 | Assay of mitochondrial respiratory chain complex activities

All enzymatic assays were performed using a 96-well plate spectrophotometer (epoch-4086) in duplicate at 37°C using a final volume of 100 µl as described in relevant publications (Ojano-Dirain, Iqbal, Wing, Cooper, & Bottje, 2005) with minor modifications. Values were corrected for the path length of the 96-well plate and with the appropriate blanks. All reagents used for the enzymatic assays were purchased from Sigma-Aldrich Co. (St. Louis, MO) or Merck Co. (Whitehouse, NJ). Enzymes activities were expressed as units (U) per milligram of mitochondrial protein.

2.4.1 | Complex I (NADH ubiquinone oxidoreductase)

Complex I (Cox I) activity was evaluated by calculating the differences between the activities in the presence and absence of rotenone. Concisely, the activity was measured by following the oxidation of NADH. Mitochondrial suspension (10 µl) was added to a solution containing 0.5 mM 2, 6 dichloroindophenol and 50 mM Tris-HCl. The reaction was initiated by adding 1 mM NADH. Absorbance of the solution was measured at 600 nm, and then, rotenone (20 µM) was added to the solution followed by incubation at 37°C for 90 s. Absorbance was measured again, and the decrease in the optical density at 600 nm was calculated. The activity was determined using an extinction coefficient of 21 mM/cm.

2.4.2 | Cox II (Succinate dehydrogenase)

The activity of Cox II was assessed as a decrease in absorption at 600 nm after the reduction of 2,6-dichlorophenolindophenol in the presence of NADH. Isolated mitochondria (10 µl) were loaded in a medium containing 10 mM KH_2PO_4, pH 7.5, 20 mM sodium succinate, 1.5 mM KCN and 1 mM NADH. The absorbance change was determined after addition of 100 µM 2,6-dichlorophenolindophenol, and then, the solution was incubated at 37°C for 3 min. After 3 min, the reduction in absorbance at 600 nm was measured and the enzyme activity was quantified using an extinction coefficient of 21 mM/cm.

2.4.3 | Cox III (Ubiquinol cytochrome *c* reductase)

Cox III activity was evaluated by monitoring cytochrome *c* reduction using decylubiquinol at 550 nm. In summary, mitochondria (10 µl) were pre-incubated at 37°C in an assay medium (pH 7.5, 2.5 mg/ml of BSA, 35 mM KH2PO4, 125 µM oxidized cytochrome *c*, 5 mM $MgCl_2$, 1.8 mM KCN and 10 µg/ml of rotenone) for 30 s. The reaction was initiated by addition of 31.8 µM reduced ubiquinone 2, and the enzyme activity was recorded for 3 min in the presence and absence of 10 mg/ml of antimycin A. The enzymatic reduction of cytochrome *c* was considered as the specific activity of Cox III. The enzyme activity was quantified using an extinction coefficient of 19.2 mM/cm.

2.4.4 | Cox IV (Cytochrome *c* oxidase)

The mitochondria (10 µl) were incubated in 10 mM KH_2PO_4, pH 7.4, at 37°C for 3 min, and then, reduced cytochrome *c* (92% reduced by using dithionite sodium) was added. The decrease in absorbance at 550 nm was measured by following the oxidation of cytochrome *c* for 90 s. The enzyme activity was calculated using an extinction coefficient of 21 mM/cm.

2.5 | Statistical analysis

The experiment was conducted in a completely randomized design, in a 2 × 2×3 × 2 split-plot factorial arrangement for all responses data, except for growth performance during 1–10 days. During this period, both houses had the same temperature, thus, the data were analysed using complete block design in 2 × 3 × 2 factorial arrangement (Met source, Met level and Bet replacement respectively). Statistical significance of differences between treatments means was determined using Tukey test (SAS Institute, 2004). All statements of significance were based on a $p < 0.05$.

3 | RESULTS

3.1 | Growth performance

The effects of dietary treatments on growth performance of the birds reared under normal condition during 1–10 days and under

normal and heat stress conditions at 11–42 days of age are shown in Tables 3 and 4. There were significant interactions between Met level and Met source for FCR ($p = 0.04$, Figure 1) and a tendency for an interaction for WG ($p = 0.051$). Birds fed diets containing 70% Met requirements from l-Met source showed better FCR than those fed diets supplemented with 70% Met requirements from dl-Met source. We did not observe interactions among other groups ($p \geq 0.05$). Met level affected broilers performance during 1–10 days. Broilers fed 70% Met diet consumed more feed than other groups, and FI of birds fed 130% Met was significantly less than other two groups ($p < 0.001$, Table 3). Also, 70% Met diet significantly decreased WG and increased FCR comparing to Req Met and 130% Met levels respectively ($p < 0.001$). According to the results presented in Table 3, the effect of main plot (house 1 and 2) on FI, WG and FCR was not significantly different ($p \geq 0.05$). This may confirm that the conditions of both houses were same during 1–10 days.

TABLE 3 Effects of dietary methionine (Met) level and source and betaine replacement on performance in broilers grown in normal condition during 1–10 days of age[1]

Treatment	Feed intake (g/bird/days)	Weight gain (g/bird/days)	Feed conversion ratio (g:g)
Main plot[2]			
House 1	22.34	18.28	1.24
House 2	22.00	18.60	1.20
SEM	0.223	0.186	0.01
Met source			
dl-Met	22.15	18.23	1.23
l-Met	22.19	18.65	1.22
SEM	0.221	0.185	0.01
Met level[3]			
70% Met	23.33[a]	16.82[b]	1.41[a]
Requirement Met	22.05[b]	19.19[a]	1.16[b]
130% Met	21.13[c]	19.30[a]	1.11[b]
SEM	0.275	0.224	0.01
Betaine[4]			
+Betaine	22.01	18.36	1.21
−Betaine	22.33	18.51	1.24
SEM	0.226	0.183	0.01
Source of variation	***p*-value**		
Main plot	0.295	0.216	0.113
Met source	0.889	0.109	0.847
Met level	<0.001	<0.001	<0.001
Betaine	0.316	0.566	0.180
Met source × Met level	0.906	0.051	0.045
Met level × betaine	0.299	0.958	0.536
Met source × betaine	0.790	0.979	0.790

Notes. Means without common superscripts "a, b" within a column are significantly different ($p < 0.05$).
[1]Each of the four-factor combinations had five replicate pens of 10 birds each ($r = 5$). [2]Birds were raised under identical temperatures at the both houses during 1–10 days. [3]70% Met; methionine level was 30% lower than Ross 308 recommendation, 130% Met; methionine level was 30% more than Ross 308 recommendation. [4]Betaine was or was not substituted at the rate of 30% of supplemental dl- or l-methionine.

TABLE 4 Effects of dietary methionine (Met) level and source and betaine replacement on performance in broilers grown in normal and heat stress conditions during 11–42 days of age[1]

Treatment	Feed intake (g/bird/days)	Weight gain (g/bird/days)	Feed conversion ratio (g:g)
Temp			
Heat stress	117.7[b]	63.54[b]	1.86[a]
Normal	121.7[a]	67.94[a]	1.80[b]
SEM	0.724	0.343	0.007
Met source			
dl-Met	119.7	65.36	1.84
l-Met	119.7	66.12	1.82
SEM	1.040	0.652	0.011
Met level[2]			
70% Met	121.4[a]	59.62[b]	2.04[a]
Requirement Met	121.4[a]	69.32[a]	1.75[b]
130% Met	116.3[b]	68.28[a]	1.70[c]
SEM	1.273	0.799	0.014
Betaine[3]			
+Betaine	119.7	66.11	1.82
−Betaine	119.7	65.37	1.84
SEM	1.040	0.652	0.011
Source of variation	***p*-value**		
Temp	0.018	0.0008	0.003
Met source	0.969	0.412	0.103
Met level	0.006	<0.001	<0.001
Betaine	0.992	0.424	0.262
Temp × Met source	0.848	0.523	0.157
Temp × Met level	0.934	0.716	0.764
Temp × betaine	0.957	0.788	0.557
Met source × betaine	0.997	0.953	0.912
Met source × Met level	0.999	0.405	0.068
Met level × betaine	0.997	0.944	0.864

Notes. Means without common superscripts "a, b" within a column are significantly different ($p < 0.05$).
[1]Each of the four-factor combinations had five replicate pens of 10 birds each ($r = 5$). [2]70% Met; methionine level was 30% lower than Ross 308 recommendation, 130% Met; methionine level was 30% more than Ross 308 recommendation. [3]Betaine was or was not substituted at the rate of 30% of supplemental dl- or l-methionine.

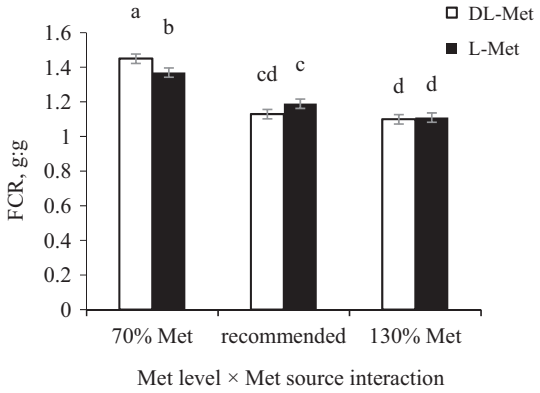

FIGURE 1 Interaction effect of methionine (Met) level and source on feed conversion ratio in broilers fed diet containing methionine in normal conditions during 1–10 days of age. 70% Met; methionine level was 30% lower than Ross 308 recommendation, 130% Met; methionine level was 30% more than Ross 308 recommendation. Each of the two factor combinations had five replicate pens of 10 birds each. Values are means, with their standard deviations represented by vertical bars. [a,b]Means without common superscripts within a column are significantly different ($p < 0.05$)

There was no significant difference between replaced or non-replaced Bet diets in the case of FI, WG and FCR during 1–10 days ($p \geq 0.05$).

During 11–42 days of age, there was a tendency for interaction between Met level and Met source for FCR ($p = 0.068$). Met level affected broilers FI ($p = 0.006$) and WG (<0.001). FI and WG of broilers fed 130% Met diet were significantly decreased and increased comparing to other two groups during 11–42 days respectively. Birds receiving 130% Met diet showed significantly lower FCR than 70% Met and Req Met diets (Table 4, $p < 0.001$). Met source and Bet-supplemented or non-Bet-supplemented diets did not significantly affect broilers performance ($p \geq 0.05$). Heat stress decreased FI (3.2%) and WG (6.4%) and increased FCR (3.3%) compared to birds reared under normal condition (Table 4).

3.2 | Mitochondrial complex activity

The changes in mitochondrial function in breast muscle tissue in heat stress and normal conditions were evaluated by examining the activity of key enzymes in respiratory complexes I, II, III and IV that are shown in Table 5. The activities of Cox I and III were influenced by the interaction between temperature and Met levels (Table 5). Broilers fed Req Met level diet under normal condition exhibited higher activities of Cox I ($p < 0.001$) and III ($p = 0.032$) compared to their counterpart in heat stress condition (Figure 2). Also, an interaction was observed between temperature and Bet for Cox I activity (Table 3, $p = 0.046$). Cox I activity in bids fed Bet-supplemented diets had no significant difference under normal and heat stress conditions; but birds fed with non-Bet-supplemented diet showed significant difference under the both conditions (Figure 3). Met level and Met source affected Cox I and

TABLE 5 Effects of dietary methionine (Met) levels and sources and betaine replacement on respiratory chain complexes activity (Cox, U/mg of mitochondrial protein) in breast muscle of broilers at 42 days of age, grown in normal and heat stress conditions[1]

Treatment	Cox I	Cox II	Cox III	Cox IV
Temp				
Heat stress	14.24[b]	29.90	14.97[b]	22.80[b]
Normal	15.94[a]	29.53	15.81[a]	26.21[a]
SEM	0.259	0.152	0.105	0.487
Met source				
dl-Met	14.73[b]	29.48	15.10[b]	24.65
l-Met	15.46[a]	29.96	15.69[a]	24.35
SEM	0.239	0.361	0.177	0.277
Met level[2]				
70% Met	12.11[c]	29.94	13.47[b]	23.90
Requirement Met	15.69[b]	29.66	16.23[a]	25.01
130% Met	17.48[a]	29.56	16.47[a]	24.59
SEM	0.293	0.442	0.217	0.339
Betaine[3]				
+Betaine	16.53[a]	29.78	15.68[a]	24.46
−Betaine	13.66[b]	29.66	15.11[b]	24.55
SEM	0.239	0.361	0.177	0.275
Source of variation	***p*-value**			
Temp	0.009	0.183	0.011	0.015
Met source	0.031	0.348	0.022	0.444
Met level	<0.001	0.824	<0.001	0.074
Betaine	<0.001	0.813	0.027	0.828
Temp × Met source	0.179	0.271	0.399	0.249
Temp × Met level	<0.001	0.766	0.032	0.475
Temp × betaine	0.046	0.511	0.691	0.668
Met source × betaine	0.599	0.961	0.791	0.699
Met source × Met level	0.200	0.277	0.360	0.218
Met level × betaine	0.839	0.543	0.777	0.398

Notes. Means without common superscripts "a, b" within a column are significantly different ($p < 0.05$).
[1]Each of the four-factor combinations had five replicate pens of 10 birds each ($r = 5$). [2]70% Met; methionine level was 30% lower than Ross 308 recommendation, 130% Met; methionine level was 30% more than Ross 308 recommendation. [3]Betaine was or was not substituted at the rate of 30% of supplemental dl- or l-methionine.

Cox III activities ($p < 0.05$). Supplementation of l-Met increased Cox I and III activities comparing to dl-Met supplementation (Cox I = 15.46 and Cox III=15.69 U/mg of mitochondrial protein). As shown in Table 5, Cox I activity was increased as the level of Met was increased. So that, the complexes activities were significantly different among three Met levels ($p < 0.001$). Birds fed 70% Met diet showed lower Cox III activity comparing to birds fed 130% and Req Met diets. Also, birds fed diet supplemented with Bet exhibited higher Cox I ($p < 0.001$) and Cox III ($p = 0.027$) activities than

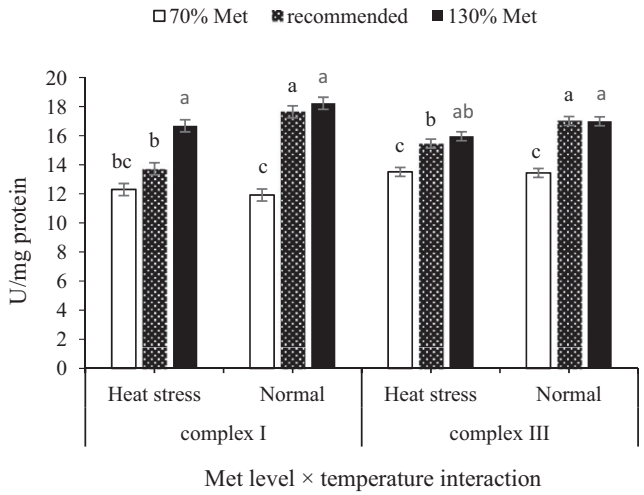

FIGURE 2 Interaction effect of methionine (Met) level and temperature on complexes I and III activity (U/mg of mitochondrial protein) in breast muscle of broilers at 42 days of age fed diet containing methionine in normal and heat stress conditions. 70% Met; methionine level was 30% lower than Ross 308 recommendation, 130% Met; methionine level was 30% more than Ross 308 recommendation. Each of the four-factor combinations had five replicate pens of 10 birds each. Values are means, with their standard deviations represented by vertical bars. [a–c]Means without common superscripts within a column are significantly different ($p < 0.05$)

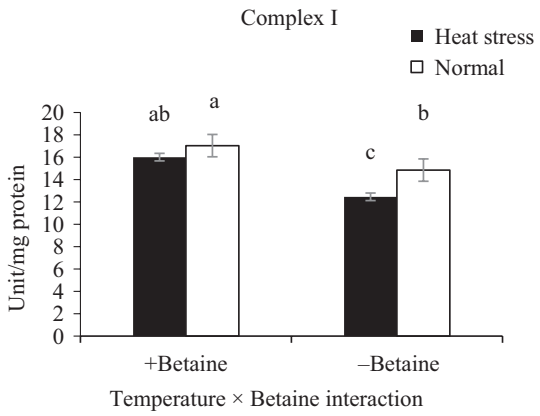

FIGURE 3 Interaction effect of betaine replacement and temperature on complexes I activity (Cox, U/mg of mitochondrial protein) in breast muscle of broilers at 42 days of age fed diet containing methionine and betaine in normal and heat stress conditions. Each four-factor combinations had five replicate pens of 10 birds each. Values are means, with their standard deviations represented by vertical bars. Betaine was or was not substituted at the rate of 30% of supplemental dl- or l-methionine. [a–c]Means without common superscripts within a column are significantly different ($p < 0.05$)

those fed non-Bet-supplemented diet. Heat stress decreased Cox I, III and IV activities ($p < 0.05$), while Cox II activity was not affected by heat stress ($p \geq 0.05$). The activities of Cox II and IV were not influenced by Met levels, Met sources and Bet replacement in the breast muscle mitochondria ($p \geq 0.05$).

4 | DISCUSSION

4.1 | Growth performance

Previous studies indicate that feeding chickens with Met deficient diets increase Betaine–Homocysteine Methyltransferase (BHMT) activity (Emmert, Garrow, & Baker, 1996). This enzyme specifically catalyses the transport of the derived methyl group from the Bet molecule to homocysteine that can be irreversibly converted to cysteine for body protein synthesis or re-methylated by other methyl sources to form Met. Therefore, similar growth performance of chickens fed with diets that their 30% supplemental Met was or was not replaced with Bet in this study indicates possibly substitution of Bet for Met. Saunderson and Mckinlay (1990) did not observe any difference in body weight between broilers fed diets supplemented with dl-Met or dl-Met + Bet. The results achieved here were not in agreement with other studies that found no positive effect of Bet supplementation on performance (Pillai, Fanatico, Beers, Blair, & Emmert, 2006; Rafeeq, Pasha, Rashid, Hilal, & Shahzad, 2011). Generally, these findings suggest that the effect of Bet supplementation on feed efficiency may depend on various factors including diet composition, feeding management, the health condition of the experimental birds, and dietary level of Met as well as supplemental Bet.

In the present study, the highest WG and lowest FCR were observed in the birds fed with 130% Met requirements, which is tendentiously due to the stimulating effect of Met on growth by means of growth factors and its influence on protein synthesis and breakdown (Kimball & Jefferson, 2006; Tesseraud et al., 2007). In contrast to our findings, Kauomar, Farhoomand, and Ebrahzadehim (2011) reported that dietary Met supplementation in practical range is usually associated with a better performance of broilers. In current study, the results in regard of growth performance are in agreement with those reported previously (Del Vesco, Gasparino, Oliveira Neto, Marcelo Rossi, et al., 2013; Vesco et al., 2015).

One of the objectives of the present study was to compare the growth responses of broilers fed diets supplemented with either l-Met or dl-Met. We found no significant differences between the two sources of Met on broilers growth performance. Our data provided evidence to support earlier results indicating that the effectiveness of dl-Met is similar to that of l-Met in chicks (Chung & Baker, 1992; Dilger & Baker, 2007). Fang et al. (2010) stated that the conversion of d-Met to l-Met is not a limiting factor due to the existence of substantial d-amino acid oxidase activity in all along the GIT, the kidney and the muscles and the genes responsible are activated in the presence of this molecule. However, there are some reports showing that l-Met is better utilized by broiler chicks compared to d-Met (Shen et al., 2015; Shen, Weaver, & Kim, 2014).

The results presented in Table 3 revealed a significant interaction between Met sources and Met levels for FCR and a tendency for an interaction for WG during 1–10 days. The birds fed 70% Met

requirements from l-Met source showed significantly lower FCR than those fed 70% Met requirements from dl-Met source (Figure 1). Shen et al. (2015) demonstrated that the bioavailability of dl-Met is about 71.5% of l-Met when daily weight gain and G: F ratio were used as the responses for very young broiler chickens. In other words, l-Met is more readily utilized by young broiler chicks than d-Met, since l-Met is the form that is directly used to synthesize body proteins, whereas d-Met has to be converted to l-Met in vivo prior to incorporation into body proteins. The inconstancy observed in bioavailability of Met isomers among studies can partly be attributed to differences in the ages of animals, because the expression of d-amino acid oxidase is very low in young animals (Shen et al., 2015). Majority of studies that indicate l-Met and dl-Met have similar bioavailabilities have been carried out with chicks at 8–20 days of age or older (Dilger & Baker, 2007), whereas in the current study the treatment were applied from day 1 of age.

In this study, diets with or without Bet replacement for Met did not show significant difference in the case of growth performance parameters during 11–42 days. Therefore, Bet could spare 30% of supplemental Met. The ability of Bet for partial sparing of Met has previously been investigated in broiler diets; however, the reported results were controversial. Studies have shown that the response to dietary Bet was greater than that obtained from Met when basal diet (formulated to provide 75% of the NRC recommendation for Met) was reinforced with Met at 0.05%, 0.10% or 0.15% or with Bet at the same level (Virtanen & Rosi, 1995). In the present study, we observed that Met and Bet supplementation to slightly Met deficient broiler diets could result in an equivalent growth response and that Met could be slightly spared by Bet. It has been reported that the osmoprotectant action of Bet alleviates the effects of heat stress and of acid-base balance changes that may disturb physiological and metabolic functions, and consequently, broiler performance and feed efficiency (Honarbakhsh, Zaghari, & Shivazad, 2007). During heat stress, the birds expend relatively large amounts of energy to control water balance. Bet helps the bird retain water more efficiently allowing more energy for growth. Therefore, factors such as the duration and extent of heat stress, the growth stages and species of broilers, and the type of diets can help to explain the differences of these results. There were no interaction between Bet with Met level, Met source and ambient temperature. Zulkifli, Mysahra, and Jin (2004) showed that Bet supplementation has no significant effect on performance of broilers reared under heat stress condition. In contrast, Attia, Hassan, Shehatta, and Abd-El-Hady (2005) found that Bet supplementation improves FCR under heat stress condition compared to control birds. Attia, Hassan, and Qota (2009) revealed that adding 1-kg Bet/ton to chickens diets could partially overcome the detrimental effects of heat stress, and improved WG and FCR compared to control group.

High ambient temperatures have deteriorative effects on broilers performance. These effects can be attributed to the heat-induced physiological changes in the bird's body. Animals suffering from heat stress experience a major metabolic burden (Willemsen et al., 2011). Bird primarily uses a series of strategies, including a reduction in FI and increase in water intake to reduce metabolic heat production and increase heat dissipation (Mujahid, Yoshiki, Akiba, & Toyomizu, 2005). In the present study, birds reared under heat stress condition had lower FI than their counterpart grew in the normal environmental condition. These findings are in agreement with those reported by Bartlett and Smith (2003). These authors demonstrated that broilers exposed to cyclic or chronic heat stress (23.9–37°C) ate less feed compared to those reared under thermoneutral zone (23.9°C).

Studies indicated that growth performance and FCR had been deteriorated when birds experienced high environmental temperatures. Heat stress disturbs the intestinal microflora balance and thereby diminishes nutrient digestibility and absorption. Broilers exposed to high ambient temperatures display higher plasma corticosterone level, leading to a sharp elevation in the breakdown of body proteins (He, Zhao, Dai, Liu, & Bokhari, 2015). Ganesan, Anandan, and Thandayan Lakshmanan (2011) also suggested that the body-weight loss is more likely due to a release of hormones such as thyroid-stimulating hormone, resulting in an increased metabolic rate, followed by a decrease in body weight.

4.2 | Mitochondrial complex activity

The activities of complexes I and III in broilers fed 70% Met requirements were lower than other groups, while Cox II and IV activities were not affected by Met level (Table 5). It was found, in several studies, that dietary nutrient composition and intake rates can influence the energetic efficiency of mitochondrial function (Dumas et al., 2004; Iossa et al., 2003). Part of these results may be assumed to be due to the effect of Met on cells mitochondria since perturbations in the physiological function of mitochondria inevitably disturb metabolism that leads to the reduction in chickens' growth and even death (Mujahid, Akiba, & Toyomizu, 2006). In agreement with these results, Yang et al. (2015) inferred that Met restriction reduces Cox I activity in porcine liver mitochondria without affecting other complexes. As stated by Del Vesco et al. (2014), ambient temperature and Met supplementation affect H_2O_2 production such that in heat-stressed quails and those fed a diet void of Met supplementation, a higher rate of H_2O_2 production was observed. They suggested that under heat stress conditions where H_2O_2 production was the highest, Met supplementation could attenuate ROS-induced damage, possibly due to the increased antioxidant activity of GSH and glutathione peroxidase.

Met is engaged in the defence mechanisms against free radicals, it participates in the biosynthesis of GSH via transmethylation–transsulfuration pathway, where catabolized to cysteine. It was shown that under different nutritional and pathological conditions, increasing the supply of cysteine or cysteine precursors via oral or intravenous administration enhances GSH synthesis and prevents GSH deficiency (Townsend, Tew, & Tapiero, 2003). On the other hand, positive correlation between reduced GSH (an important mitochondrial antioxidant) and activities of Cox II, IV and V have been reported by Ojano-Dirain et al. (2005) who indicated antioxidant

protection is important for optimal activity of the electron transport chain. Therefore, it is reasonable to assume that an increase in Cox activities is attributed to decrease ROS-induce damage to the protein complexes via enhancing GSH synthesis in diets containing high Met. Del Vesco, Gasparino, Oliveira Neto, Guimarães, et al. (2013) illustrated that Met supplementation does not influence the expression of COX III mRNA. The mitochondria of rats fed with a Met deficient diet showed higher oxidative phosphorylation, with an increase in the activity of cytochrome oxidase. However, the correlation between the activity of cytochrome oxidase and efficiency of oxidative phosphorylation (ATP/O, amount of ATP production per amount of oxygen consumption) was found to be negative. The results of the later study indicated that feeding Met-restricted diets to animals, the inefficiency of the proton pump at the COX level may reduce the efficiency of mitochondrial ATP synthesis and increase in electron leakage (Romestaing et al., 2008).

Previous reports have shown that Met restriction reduces the concentrations of mitochondrial complexes and decreases mitochondrial ROS generation in rodents, which shows a regulatory effect of Met on mitochondrial electron transport system (Gomez et al., 2011; Sanchez-Roman & Barja, 2013). It is reported that after Met restriction in rat brain (Naudí et al., 2007) and liver (Caro et al., 2008), Cox III was also decreased. Similar results showed that the level of protein complexes I, II and III in the respiratory chain did not vary in rat liver mitochondria of Met supplemented and control group, whereas Gomez et al. (2009) stated that the level of Cox IV was significantly decreased in the Met supplemented group. In another research, the level of complexes I and IV (Ayala et al., 2007) in rat heart and liver mitochondria was decreased by Met restriction.

It is worth point out that most of the researches on comparing d-Met with l-Met were conducted on performance parameters, while there is a little information at the organelle level. In the present study, birds fed a diet supplemented with l-Met had higher complexes I and III activities compared with those fed a diet supplemented with dl-Met that underlying mechanisms remain largely unknown. A potential explanation for the difference between mitochondrial Cox respiratory enzyme activities of different Met sources may be the lower affinity of transporter systems for d-isomer absorption rather than the l isomer (Dilger & Baker, 2007). Chen et al. (2015) reported that l-Met had greater efficiency for GSH production in the GIT; since l-Met is the only biologically functional form of Met and d-Met in dl-Met is not capable to be used in the GIT unless it is converted to l-Met in the liver or kidney.

Bet supplementation, as well as Met, increased complexes I and III activities without significant effect on other complexes ($p \geq 0.05$). Recently, the direct effect of betaine on mitochondrial energy metabolism (Lee, 2015) and its protective effects of on mitochondrial function and mitochondria-mediated cell death process have been revealed in several experimental models (Ganesan et al., 2007; Garrett et al., 2013; Nash, Paleg, & Wiskich, 1982). All these data showed that mitochondrial protecting properties of betaine could play a role in its cytoprotection mechanism. We assumed that these changes are associated with the protective role of Bet from nitric oxide generation and normalization of SAM: SAH ratio and the maintenance of methylation potential in response to Bet supplementation in heat-stressed birds. Lee (2015) showed that Bet treatment leads to an upregulation of mitochondrial and intact cellular respiration and cytochrome *c* oxidase activity in human H2.35 cells. Also, it was suggested that Bet induces the activation of AKT that stimulates mitochondrial biogenesis, oxidative phosphorylation complexes and ATP generation in cardiac and skeletal muscles. It is, therefore, possible that the improved mitochondrial Cox activity after Bet treatment might be mediated by the AKT signalling pathway. Bet preserves the functioning of the electron transport chain, while the exact mechanism for this protection at the organelle level is not known. Literally, at concentrations that maintain methylation reactions, supplementation with methyl donors preserve mitochondrial proteome, mitochondrial ribosome dissociation, and increase mitochondrial superoxide production, and mitochondrial DNA damage (Sykora, Kharbanda, Crumm, & Cahill, 2009). Although, in our study, the activities of complexes I and III were increased by Bet supplementation. While the protective action of Bet at the level of the mitochondria is not recognized, more studies are required to understand the mechanisms responsible for this protection. Since, Bet has many physiological functions, and data from past studies can be interpreted as inconclusive and conflicting; further researches with Bet in heat-stressed birds will be beneficial, especially if different levels of Met are tested as well.

Production of free radicals is an integral and normal feature of cellular functions, whereas excessive generation of ROS or inadequate removal of free radicals causes destructive and irreversible damage to the cells (Lopaczyski & Zeisel, 2001). In our study, high ambient temperature decreased the activities of complexes I and III which are known as major sites of ROS production (Brand, 2010). Indeed, the result suggests that heat stress may suppress activities of Cox I and III and consequently stimulates ROS production. It is well accepted that high temperature-induced stress increases ROS production and decreases MRCE activities. The production of ROS is a function of electron leakage during oxidative phosphorylation; however, the mechanism of ROS production in heat-stressed birds has not yet been completely understood (Tan et al., 2010). Protein oxidation, decreased activity of respiratory chain complexes and different forms of mitochondrial damages may be suggested as possible mechanisms of ROS production in birds under heat stress conditions. These findings neatly confirm the results reported by Tan et al. (2010) who stated that the activities of the respiratory chain complexes I + III and IV, not including complexes II + III, were inhibited significantly in a temperature-dependent pattern following acute exposure to different high ambient temperatures (32–38°C).

More recently, Huang et al. (2015) reported that heat stress suppresses the activity of Cox I in both breast and thigh muscles and elevated Cox III activity in the breast but not in thigh muscle of broiler chickens. In contrast, elevations were detected in mitochondrial biogenesis and the enzymatic activity of selected

subunits of the respiratory chain complexes in response to acute and limited exposure to stress mediators in a study by Manoli et al. (2007). These changes could be interpreted as biological efforts to meet the increased energy demands of the cell. However, excessive acute or prolonged challenges to mitochondrial homoeostasis can result in impaired respiratory chain function and decreased ATP production (Duclos et al., 2004). As Mujahid, Akiba, and Toyomizu (2009) showed in their study, the membrane potential was higher in complex IV in muscle mitochondria of heat-stressed birds compared to that of control birds. Figure 2 demonstrates that there was no significant difference between normal and heat-stressed birds in 70% and 130% Met requirements regarding to Cox I and III activity. Although, there is little information available about the effects of "chronic" heat stress on the oxidative damage to skeletal muscle of broiler chickens; however, adaptation to heat stress over the 30 days chronic study may be responsible for the lack of differences observed. Azad, Kikusato, Sudo, Amo, and Toyomizu (2010) reported that "chronic" heat stress may not induce as much oxidative damage as "acute" heat stress that stimulates ROS production and increases oxidative damage to skeletal muscle. This figure may also indicate that the level of Met requirements of the broilers for optimum activities of Cox I and III in heat stress condition is higher than Ross 308 strain Met requirement, and 130% Met requirements are suitable for heat stress condition regarding to Cox I and III activities.

The results achieved in this study indicated that heat-stressed chickens had lower WG and higher FCR compared to those reared in normal temperature condition. Stress-induced mitochondrial inefficiency is associated with the low performance of broilers (Bottje et al., 2002; Bottje, Pumford, Ojano-Dirain, Iqbal, & Lassiter, 2006). Although heat stress-induced ROS production was not directly measured in the current study, it can be assumed that the production of ROS may be induced by heat exposure via inhibiting the activity of mitochondrial respiration and therefore, compromising ATP generation. At least to some extent, this may be responsible for the retarded growth performance of heat-stressed broilers.

5 | CONCLUSION

The results from the present study showed that adequate Met and 130% Met requirements diets improved broilers growth performance, although lowest FCR was related to 130% Met requirements diet at the end of the rearing period. There was no significant difference between dl- and l-Met on growth performance of the birds. l-Met supplementation was more effective than dl-Met at the cellular level. l-Met increased Cox I and III activities that may improve ROS scavenging and may increase heat stress tolerance of the birds. Substitution of 30% supplemental Met with Bet resulted in similar growth performance comparing to non-substituted diets. Bet replacement for Met could mitigate the effects of heat stress, through an increase in the activities of respiratory chain complexes (I and III) in heat-stressed birds. High ambient temperature depressed growth performance and activity of the mitochondrial respiratory chain of broiler chickens. This study showed, for the first time, Bet could have a protective effect on mitochondrial function in tissues of broiler chickens. There is limited information about the effect of Met and Bet on the mitochondrial respiratory chain in broiler chickens exposed to heat stress. Thus, further research is needed to evaluate the effect of Met and Bet on the mitochondrial respiratory chain in broiler chickens.

ACKNOWLEDGEMENTS

The authors would like to appreciate Ferdowsi University of Mashhad for the financial support of this study (project# 3/38938). We are also grateful from Dr. Javadmanesh director of genetics laboratory and Dr. Rajaei Sharifabadi for their assistance.

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How to cite this article: Sahebi Ala F, Hassanabadi A, Golian A. Effects of dietary supplemental methionine source and betaine replacement on the growth performance and activity of mitochondrial respiratory chain enzymes in normal and heat-stressed broiler chickens. *J Anim Physiol Anim Nutr.* 2019;103:87–99. https://doi.org/10.1111/jpn.13005