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Dietary chromium-methionine supplementation and broiler (22–43 days) responses during heat stress. 2 - Physiological variables, and heat shock protein 70 and insulin-like growth factor-1 gene expression

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ABSTRACT

Context. Dietary supplementation with trace mineral chromium (Cr) has been shown to enhance the physiological responses of broilers subjected to heat stress (HS), modulate gene expression, and improve performance. **Aims.** This study aimed to evaluate the impact of chromium–methionine (CrMet) supplementation on growth performance, body temperatures, lymphoid organ weights, hormones, blood parameters, and the expression of heat-shock protein-70 (HSP-70) and insulin-like growth factor-1 (IGF-1) genes in broilers under HS conditions (33°C for 12 h/day). **Methods.** In the first experiment, 336 22-day-old male broilers were randomly distributed into four blocks with six treatments (0, 0.10, 0.20, 0.40, 0.80, and 1.20 mg/kg CrMet) and eight replicates with seven birds per cage. These broilers were subjected to HS from 22 to 43 days of age. In the second experiment, 24 male broilers, in total, at 43 days of age, previously exposed to HS, were randomly distributed to the same six treatments from the first experiment, with four replicates. Breast samples were collected for the analysis of HSP-70 and IGF-1 expression. **Results.** A quadratic effect ($P < 0.05$) was observed on bodyweight gain (BWG) and feed conversion ratio (FCR). The supplementation of 0.71 and 0.68 mg/kg improved BWG and FCR, respectively. At 28 days of age, cloacal and mean body temperatures, corticosterone, and thyroid hormones were quadratically affected ($P < 0.05$), while at 43 days of age, a linear effect ($P < 0.05$) was observed on haemoglobin concentration. There was a reduction ($P < 0.05$) in the expression of HSP-70 and an increase in IGF-1 ($P < 0.05$) in the breast tissue of broilers supplemented with CrMet. **Conclusions.** The supplementation with 0.71 mg/kg and 0.68 mg/kg of CrMet improved BWG and FCR, respectively. Additionally, the supplementation with 0.80 mg/kg improved hormones, reduced HSP-70 and increased the expression of IGF-1 in broilers during HS. **Implications.** These findings suggest that CrMet can be included in the diet of broiler chickens subjected to HS to enhance physiological responses and performance.

Keywords: animal physiology, animal production, growth performance, heat stress, hormones, mineral nutrition, organic mineral, trace mineral.

Introduction

Heat stress (HS) has a significant impact on broiler production, especially in tropical and subtropical regions (He *et al.* 2018). The adverse effects on broiler performance result from complex endocrine and metabolic physiological adjustments triggered by HS (Wasti *et al.* 2020). From these mechanisms, it can be inferred that animals regulate their feed consumption on the basis of their ability to dissipate heat generated by metabolism. As a result, environmental conditions, genetic factors, and nutrient supply play pivotal roles in optimising physiological responses (Das *et al.* 2016).

Recent literature has indicated that HS results in decreased broiler growth, compromised immune responses, and disruptions in hormone and blood metabolite concentrations, all of

which are critical factors for broiler health (Kumari and Nath 2018; Saleh *et al.* 2018; Safwat *et al.* 2020; Dalólio *et al.* 2021). Understanding these effects is essential for developing strategies to mitigate the economic losses associated with HS in the broiler industry.

Chromium (Cr) is not classified as an essential trace mineral for livestock (Vincent 2017), and it is infrequently included in monogastric premix formulation. Nevertheless, recent research suggests that Cr may play crucial nutritional and physiological role in broilers (Sahin *et al.* 2017, 2018). Chromium supplementation has demonstrated positive influence on insulin sensitivity in cells through the low-molecular-weight Cr-binding-substance (LMWCr) (Vincent, 2000, 2010). This effect stimulates cellular anabolism and influences carbohydrate, lipid, and protein metabolism, resulting in an increase in the apparent metabolisable energy corrected for nitrogen in the diet (Anderson 1997; Lu *et al.* 2019; Dalólio *et al.* 2021).

Furthermore, Cr supplementation has been shown to elevate triiodothyronine (T3) and thyroxine (T4) concentrations while reducing serum corticosterone (COR) in broilers exposed to HS, leading to improvements in their growth performance (Sahin *et al.* 2002, 2003; Bharami *et al.* 2012). Studies conducted by Ghazi *et al.* (2012) and Jahanian and Rasouli (2015) indicated that HS results in increased COR concentrations and decreased lymphatic tissue, compromising immunity and reducing broiler performance.

Heat stress in broilers promotes cellular damage, and the expression of heat-shock protein (HSP) can serve as an indicator of decreased performance in response heightened oxidative stress (Akdemir *et al.* 2015). Rajkumar *et al.* (2018) reported reduction of HSP-70 expression in heart, liver, muscle, and spleen of broilers reared under HS and fed Cr-yeast in the diet. Chromium up-regulates cell signalling to recognise insulin (Vincent, 2000, 2017). Insulin-like growth factor-1 (IGF-1) is important for protein synthesis and to decrease proteolysis in broilers, especially under HS (Sacheck *et al.* 2004). Researchers have not investigated gene expression of IGF-1 and HSP-70 in the breast of broilers exposed to HS and fed diet supplemented with chromium–methionine (CrMet).

The present study is a continuation of the results evaluated by Dalólio *et al.* (2021), where the authors investigated the effect of CrMet supplementation on growth performance and carcass yield, metabolisable energy, and serum biochemistry. Thus, the study aimed to evaluate the effect of supplementing CrMet during HS on body temperature, lymphoid tissues, hormone profiles, haemogram, and gene expression of HSP-70 and IGF-1.

Materials and methods

All procedures utilised in the present study were approved by the Ethics and Research Committee of the Federal University of Viçosa, Viçosa, Brazil (Protocol Number 15/2016). As highlighted, the current investigation extends the findings assessed by Dalólio *et al.* (2021). Accordingly, the same animals, experimental design, dietary treatments, and management procedures were utilised, while introducing variations solely in the evaluated variables.

Experiment 1

Three hundred and thirty-six 22-day-old male Cobb 500® broilers with average bodyweight (BW) of 858.20 g (±42 g) were used in the study. The birds were maintained under thermal comfort conditions from Day 1 to Day 21, following the guidelines outlined in the Cobb 500® Broiler Management Guide (Cobb-Vantress 2013). During the periods from 1 to 7, 8 to 14, and 15 to 21 days of age, ambient temperatures averaged 32.1°C, 29.8°C, and 26.9°C respectively. Birds were fed a common starter diet according to the nutritional recommendations by Rostagno *et al.* (2011) (Table 1).

During the experimental period, from 22 to 43 days of age, HS was induced by maintaining broilers in climatic chambers at a temperature of 33.0 ± 0.8°C for 12 h (7:00 am to 6:59 pm). Subsequently, broilers were subjected to a temperature of 23.0 ± 0.8°C (7:00 pm to 06:59 am). Relative humidity was maintained at 65.0 ± 3.5% throughout the entire experimental period.

Boilers had *ad libitum* access to mash feed and water, and mortality was recorded daily. The light program consisted of 20 h of artificial light and 4 h of darkness from 22 to 43 days of age, with three fluorescent lamps installed per chamber.

Experiment 1 employed a randomised complete-block design with four blocks (each block comprised a climatic chamber), six treatments, eight replicates (2 replicates per block), and seven birds per experimental unit. The treatments involved varying concentrations of CrMet (Availa® Cr 1000, Zinpro Corporation, Eden Prairie, MN, USA) in the basal diet for 22–43-day-old broilers, following nutritional recommendations by Rostagno *et al.* (2011). Specifically, the six CrMet inclusion concentrations were 0.00, 0.10, 0.20, 0.40, 0.80, and 1.20 mg/kg.

The hypothesis to assess the dosages of CrMet used in the study was based on a previously published review article by Dalólio *et al.* (2018), in which the average maximum dose of 1.099 mg/kg of Cr, regardless of the source used, was optimum to improve physiological variables.

Chemical composition of diets and Cr analysed are shown in Table 2. To determine the DM in basal diet, samples were dried at 105°C for 16 h (AOAC 2006, Method 934.01) in a drying oven. Nitrogen (N) was determined by the total combustion of sample, and the Dumas method (AOAC 2006, Method 968.06) was used to calculate the crude protein (CP) content (N × 6.25). Ether extract (EE) was determined by Soxhlet method (Method 920.39), and the ash content was measured by burning the samples at 650°C for overnight (Method 942.05). Crude fibre (CF) was determined according to AOAC (2006, Method 962.09). Calcium (Ca) and

Table 1. Ingredients and nutritional composition of experimental basal diet in the initial stage from 1 to 21 days, and from 22 to 43 days of age of broilers.

Ingredient	1 to 21 days (%)	22 to 43 days (%)
Corn	59.742	63.741
Soybean meal (45%)	34.139	30.260
Soybean oil	1.835	2.650
Dicalcium phosphate	1.717	1.156
Limestone	1.040	0.816
Salt	0.455	0.440
DL-methionine (99%)	0.235	0.209
L-lysine HCl (79%)	0.204	0.136
Choline chloride (60%)	0.100	0.100
Vitamin supplement[A]	0.100	0.100
Mineral supplement[B]	0.100	0.100
Salinomycin 12%[C]	0.055	0.055
Avilamycin 10%[D]	0.010	0.010
BHT[E]	0.010	0.010
Phytase[F]	0.007	0.007
Inert filler[G]	0.251	0.210
Total	100.00	100.00
Calculated composition		
Crude protein (%)	21.000	19.000
Metabolisable energy (kcal/kg)	2950	3100
Digestible lysine (%)	1.140	1.005
Digestible methionine + cystine (%)	0.807	0.733
Digestible threonine (%)	0.700	0.654
Calcium (%)	0.890	0.683
Non-phytate phosphorus (%)	0.420	0.319
Sodium (%)	0.210	0.190

[A]Vitamin premix for birds, guaranteed concentrations (minimum) per kilogram of feed: vitamin A 12,000 IU; vitamin D3 2200 IU; vitamin E 3.00 IU; vitamin B1 2.20 mg; vitamin B2 6.00 mg; vitamin B6 3.30 mg; pantothenic acid, 13.00 mg; biotin 0.11 mg; vitamin K3 2.50 mg; folic acid 1.00 mg; nicotinic acid 53.00 mg; niacin 25.00 mg; vitamin B12 16.00 µg.
[B]Mineral premix for birds, guaranteed concentrations (minimum) per kilogram of feed: manganese 75.00 mg; iron 200.00 mg; selenium 2.50 mg, zinc 500.00 mg; copper 40.00 mg; cobalt 2.00 mg; iodine 1.50 mg.
[C]Coccidiostat.
[D]Antibiotic growth promoter.
[E]Antioxidant.
[F]Phytase 500 FTU/kg supplemented on top.
[G]Kaolin.

phosphorus (P) were determined according to AOAC (2006, Methods 984.01 and 965.17 respectively). The Cr quantification was performed using atomic absorption spectrophotometry (Williams *et al.* 1962).

At 28 and 42 days of age, temperatures of comb, head, wattle, back, wing, breast and legs were measured using an infrared thermometer (Model TI-870, Instrutherm®, São Paulo, SP, BR).

Table 2. Chemical analysis of control diet and analysed chromium concentration in the experimental diets (as-fed basis); –, not detected.

Nutrient	Nutrient (%)	Chromium (mg/kg)	
	Analysed	Formulated	Analysed
Dry matter	90.40	0 (Control diet)	–
Crude protein	19.30	0.10	0.12
Ethereal extract	5.65	0.20	0.18
Crude fibre	2.57	0.40	0.35
Calcium	0.69	0.80	0.76
Phosphorus	0.65	1.20	1.24

Subsequently, mean skin temperature (MST, °C) was calculated using the following equation:

$$\mathrm{MST} = (0.03T_{\mathrm{crest}}) + (0.70T_{\mathrm{back}}) + (0.12T_{\mathrm{wing}}) + (0.06T_{\mathrm{head}}) + (0.09T_{\mathrm{legs}})$$

where T_{comb} = crest temperature (°C); T_{back} = back temperature (°C); T_{wing} = wing temperature (°C); T_{head} = head temperature (°C); T_{leg} = leg temperature (°C) described by Richards (1971). Cloacal temperature (CT, °C) was mensured using a digital thermometer with accuracy of ±0.1°C. Mean body temperature (MBT, °C) was calculated using the following equation described by Richards (1971):

$$\mathrm{MBT} = (0.30\mathrm{MST}) + (0.70\mathrm{CT}).$$

At Day 43, two broilers per replicate (total of 96 birds, 16 per treatment) were selected within ±5% of the pen average BW, euthanised by electronarcosis and bled by ventral neck cutting, and absolute and relative weight of bursae and spleen were measured. Relative weight of lymphoid organs was calculated as a function of carcass weight, as follows:

$$\%\text{Relative organ weight} = (\text{organ weight} \times 100/\text{carcass weight}).$$

At 43 days of age, blood samples were obtained from two broilers selected within ±5% of the pen average BW (total of 96 broilers, 16 per treatment) for analyses of T3, T4, and corticosterone hormones in addition to complete blood count (erythrograms and leukograms). Blood sample was taken by cardiac punch and stored in polyethylene tubes. After coagulation, tubes were centrifuged at 3220*g* for 15 min and 4°C temperature. Serum obtained was then stored at −80°C until hormone analyses.

Hormonal analyses were performed by radioimmunoassay (RIA) analyses by using iodine-labelled commercial kits; analyses followed supplier recommendations for each hormone. Corticosterone serum concentrations were determined by RIA by using an ImmunoChem Double Antibody Corticosterone 125I RIA Kit (MP Biomedicals LLC, Orangeburg, NY, USA). The concentrations of hormones T3

and T4 were determined using commercially available RIA kits (Byk-Sangtec Diagnostica, Dietzenbach, HE, DE).

Hematocrit was measured by microhematocrit method, in which capillary tube was centrifuged (room temperature; 20°C) at 11.360*g* for 5 min in a microcentrifuge. Mean corpuscular volume, mean corpuscular haemoglobin, erythrocyte, monocyte, eosinophil, and leucocyte counts were measured by a haemocytometer, in which blood samples were diluted to 1:200 in Natt–Herrick's dye (Vetlab, Palmetto Bay, FL, USA). Haemoglobin (Hmo) concentration was measured using the cyanmethaemoglobin method (Bioclin enzymatic kit, Bioclin, Belo Horizonte, MG, BR) by using a spectrophotometer for measuring absorbance. The differential leukocyte count was performed on blood smear, fixed with methyl alcohol (methanol) and later it was stained with fast dye for haematology (fast panoptic). From the results, the heterophile (H):lymphocyte (L) ratio (H:L) was determined for the identification of HS in broilers and its association with the immune system.

Experiment 2

At the end of Experiment 1, 24 43-day-old male Cobb 500® broilers, in total, with an average BW of 2500.50 g (±50 g), were reared under a completely randomised experimental design with six treatments and four animals per treatment, and kept at 33°C for 12 h. Treatments consisted of the basal diet with one of six CrMet concentrations (Table 1) formulated for 22–43-day-old broilers, according to nutritional recommendations of Rostagno *et al.* (2011). The six CrMet inclusion concentrations were 0.00, 0.10, 0.20, 0.40, 0.80 and 1.20 mg/kg (Availa® Cr 1000, Zinpro Corporation, Eden Prairie, MN, USA).

To investigate the gene expression of HSP-70 and IGF-1 in the *Pectoralis major* muscle, broilers were subjected to a 12-h period of HS at 33°C. Afterwards, broilers were stunned and subsequently slaughtered by bleeding and their pectoral skin was removed. Immediately after this process, a sample of approximately 2.0 cm in length, 0.5 cm in width and 0.5 cm in depth of the right *Pectoralis major* muscle was collected in the central region of the muscular part. Subsequently, identified samples were immersed in RNA holder (BioAgency) and stored in a freezer at −20°C until extraction for the analysis of HSP-70 expression and IGF-1 mRNA gene expression.

Total mRNA from each sample was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The concentration of total mRNA was determined using the Nano Vue Plus (GE Healthcare) spectrophotometer. The integrity of RNA was assessed using 1% agarose gel and visualised by ultraviolet light. The cDNA was synthesised using the SuperScriptTM III First-Strand Synthesis Super Mix kit (Invitrogen). Volumes of 6 μL of the total mRNA, 50 μM oligo (dT)20, and 1 μL of annealing buffer were added. The mixture was incubated for 5^^min at 65°C and then placed on ice for 1 min. After, 10 μL of 2× First-Strand Reaction Mix solution and 2 μL of SuperScript III reverse transcriptase enzyme were added. Samples were stored at −20°C until analysis.

Gene sequences were obtained from the NCBI database (www.ncbi.nlm.nih.gov). Table 3 displays the sequences of the primer set. The primers were designed according to sequences obtained from GeneBank, by using the PrimerQuest Tool (www.idtdna.com/Primerquest/Home/Index) available on the IDT platform (www.idtdna.com). Two endogenous controls, the *β-actin* and *GAPDH* genes, were used. The *GAPDH* gene was used as endogenous control because there was less variation in relation to *β-actin*, when the GeNorm program was used (Vandesompele *et al.* 2002). All assays were performed in a final volume of 25 μL and in duplicate.

The fluorescence compound SYBR® Green PCR Master Mix (Qiagen) was used for real-time polymerase chain reaction (RT-PCR). The RT-qPCR reaction was run by using the SYBR Green detection kit with GoTaq qPCR Master Mix (Promega, Madison, WI, USA), using specific primers. Gene amplification was performed in duplicate by using the Quick Time PCR 7500 Fast (Applied Biosystems, Foster City, CA, USA) and the results were obtained with the Sequence Detection Systems program (V.2.0.6) (Applied Biosystems, Foster City) that generated the parameter cycle threshold (Ct). The Ct values were exported to Microsoft Excel to calculate the Ct mean, standard deviation, and standard curve for each gene. A negative control (distilled water) was also added in each assay. The qPCR reaction conditions were defined as follow: initial denaturation at 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 s. The temperature range between 60°C and 64°C for 1 min was ideal for all primers.

Statistical analyses

Data were subjected to variance and regression analyses. The residuals from the models for all variables were submitted to assumption tests. Residual normality was verified on the basis of visualisation of histograms, quantile–quantile plots, and the Shapiro–Wilk's test. Residual independence was verified through graphics of predicted versus residual values.

Homogeneity of variances was verified through box-plot graphs and the Levene's test. In general, all the assumptions

Table 3. Sequence of primers HSP-70, IGF-1, *β-actin*, and *GAPDH*.

Gene	GeneBank ID	Sequence 5′ → 3′	Product size (bp)
HSP-70	J02579	CGTGACAATGCTGGCAATAAGCGA TCAATCTCAATGCTGGCTTGCGTG	95
IGF-1	M32791	CACCTAAATCTGCACGCT CTTGTGGATGGCATGATCT	191
β-actin	NM_205518	AGACATCAGGGTGTGATGGTTGGT TCCCAGTTGGTGACAATACCGTGT	150
GAPDH	NM_204305	CCCAGCAACATCAAATGGGCAGAT TGATAACACGCTTAGCACCACCCT	133

were met for all the variables with some exceptions. When an assumption was not met, an exclusion of extreme values was performed on the basis of the standardised residuals, where values outside the range of ±3 were excluded (representing 3 standard deviations from the mean, which is zero). Such an approach solved the problems and resulted in meeting the assumptions.

Following, the regression effects (linear and quadratic) were tested using orthogonal contrasts. When a quadratic effect was obtained, the point of maximum/minimum dietary CrMet concentration was calculated. After performing the linear regressions for all the variables, non-linear regressions were performed when the variables presented *P*-values < 0.10 with linear (LBL) and quadratic. When linear polynomial regressions or non-linear regressions (LBL and QBL) were significant, the coefficient (R^2) and root mean squared error (RMSE) were presented for comparisons. All the analyses were performed in the SAS statistical software, version 9.0 (SAS Institute, Inc., Cary, NC, USA, 2012). ANOVA, regression, and orthogonal contrasts were performed using the SAS PROC MIXED. Assumptions of residual normality, homogeneity and independence were made using SAS PROC UNIVARIATE, SAS PROC GLM and SAS PROC REG, respectively. Non-linear regression models (LBL and QBL) were performed using SAS PROC NLIN. Significance was considered at the level of $P < 0.05$ (5%) of probability.

In Experiment 2, to perform statistical analyses of gene expression data, a %QPCR_MIXED macro (Steibel *et al.* 2009) was used in SAS statistical software, version 9.0 (SAS Institute, Inc., Cary, NC, USA, 2012). This macro analyses data were performed using a mixed linear models of RT–qPCR data. This analysis standardises data by using the method ΔΔCT (Livak and Schmittgen, 2001), generating contrasts which are the differences between the control and other treatments (Fu *et al.* 2006). To determine differences among treatments (CrMet levels), contrast analyses were performed. In this statistical model, CrMet concentrations (0; 0.10; 0.20; 0.40; 0.80 and 1.20 mg/kg) were considered a fixed effect, and genes were considered random effect. In this way, all possible comparisons between levels of these factors were tested. Statistical differences were set at $P < 0.05$.

Results

No Cr was detected in the control diet (Table 3). The analysed Cr concentrations in the experimental diets were close to the calculated concentrations for the experimental treatments.

Table 4 presents the effects of increasing dietary concentrations of CrMet on growth performance of heat-stressed broiler chickens from 22 to 43 days of age. The results showed that there were no significant effects on feed intake (FI) ($P > 0.05$). However, a quadratic effect was observed on BWG ($P = 0.01$) and FCR ($P < 0.01$).

Table 4. Mean values of feed intake (FI), bodyweight gain (BWG), and feed conversion ratio (FCR) of heat-stressed broiler chickens from 22 to 43 days of age.

Variable	Concentration of CrMet (mg/kg)						s.e.m.	*P*-value	
	0	0.10	0.20	0.40	0.80	1.20		L	Q
FI	2847	2806	2831	2837	2824	2846	19.42	0.52	0.43
BWG	1471	1571	1545	1589	1530	1561	18.92	0.06	0.01
FCR	1.94	1.79	1.84	1.79	1.85	1.82	0.03	0.11	<0.01

P-values show the significance of the regression effects from orthogonal contrasts.

Table 5. Mean values of the cloacal temperature (CT), skin temperature (MST) and body temperature (MBT) of broilers at 28 and 42 days of age.

Item	Concentration of CrMet (mg/kg)						s.e.m.	*P*-value	
	0	0.10	0.20	0.40	0.80	1.20		Linear	Quadratic
28 days									
CT (°C)	41.87	40.81	42.05	42.15	41.99	41.81	0.13	0.72	0.03
MST (°C)	36.60	36.98	36.36	35.60	36.49	35.99	0.43	0.13	0.31
MBT (°C)	40.38	40.36	40.34	40.18	40.34	39.97	0.19	0.04	0.54
42 days									
CT (°C)	42.60	42.63	42.24	42.30	42.61	42.48	0.23	0.97	0.56
MST (°C)	33.74	33.53	33.78	34.30	33.98	33.73	0.45	0.70	0.11
MBT (°C)	39.94	39.90	39.70	39.86	40.02	39.85	0.20	0.83	0.92

P-values show the significance of the regression effects from orthogonal contrasts.

Table 5 shows the effects of dietary CrMet on broiler body temperature variables. At Day 28, there was a quadratic effect ($P = 0.03$) of CrMet supplementation on cloacal temperature and a linear decrescent effect ($P = 0.04$) on mean body temperature. There were no significant ($P > 0.05$) effects of CrMet supplementation on mean skin temperature at Day 28, and on cloacal temperature, mean body temperature, and mean skin temperature at 42 days of age.

Table 6 shows the effects of increasing dietary concentration of CrMet on lymphoid organs of broilers. The supplementation of CrMet did not have a statistically significant ($P > 0.05$) effect on the absolute and relative weights of lymphoid organs at Day 43.

The effects of the increasing dietary concentration of CrMet on blood profile of broilers are presented on Table 7. There was a significant ($P = 0.01$) linear effect of supplemented CrMet on mean corpuscular haemoglobin concentration at Day 43. There were no significant ($P > 0.05$) effects on the other blood variables analysed.

Effects of the increasing dietary concentration of CrMet on hormones of broilers are presented on Table 8. There was a quadratic effect of CrMet supplementation on serum COR ($P < 0.01$), T3 ($P < 0.01$), and T4 ($P < 0.01$) of broilers at

Table 6. Mean values of absolute weight and relative weight of lymphoid organs of broiler chickens slaughtered at 43 days of age.

Variable	Concentration of CrMet (mg/kg)						s.e.m.	*P*-value	
	0	0.10	0.20	0.40	0.80	1.20		Linear	Quadratic
Absolute (g)									
Spleen	1.53	1.85	2.01	1.75	1.77	1.93	0.12	0.27	0.73
Bursa	3.15	3.72	3.26	3.30	3.64	3.58	0.28	0.35	0.90
Relative (%)									
Spleen	0.08	0.09	0.11	0.09	0.09	0.10	0.01	0.30	0.64
Bursa	0.16	0.19	0.17	0.17	0.19	0.19	0.01	0.36	0.85

P-values show the significance of the regression effects from orthogonal contrasts.

43 days of age. There was no significant ($P > 0.05$) effect of CrMet supplementation on the T3:T4 ratio.

The equations for each variable, with the respective adjusted model are presented in the Table 9. Considering the growth performance, the QR model estimated concentrations of 0.71 and 0.68 mg/kg of CrMet to optimise BWG and FCR respectively.

For the body temperature variables, QR model estimated the concentration of 0.57 mg/kg DM Cr as CrMet supplemented for cloacal temperature at 28 days of age.

At Day 43, for serum COR concentrations, the QR, LBL, and QBL equations estimated supplemented CrMet concentrations of 0.75, 0.23, and 0.29 mg/kg respectively. The LBL model was selected as the best fit because of its higher R^2 and RMSE values, as well as the lower CrMet inclusion in the diets required to achieve the desired reduction in serum COR. In terms of serum T3 concentrations at Day 43, the estimated supplemented CrMet concentrations by the QR, LBL, and QBL equations were 0.68, 0.10, and 0.14 mg/kg respectively. The LBL model was the preferred choice owing to its higher R^2 and RMSE values and its lower CrMet supplementation requirement to attain higher serum T3 concentrations. Regarding serum T4 concentrations, the LBL and QBL equations estimated supplemented CrMet concentrations of 0.15 and 0.22 mg/kg, respectively. The LBL model was chosen, because of its higher R^2 and RMSE values and the lower CrMet content needed in the broiler diets to elevate T4 serum concentrations.

Effects of the increasing dietary concentrations of CrMet on HSP-70 and IGF-1 expression are presented on Table 10.

Table 7. Mean values of the blood variables of broilers at 43 days of age.

Haemogram	Concentration of CrMet (mg/kg)						s.e.m.	*P*-value	
	0	0.10	0.20	0.40	0.80	1.20		Linear	Quadratic
Ery (10^6 µL)	1.99	2.12	2.04	1.84	2.26	2.15	0.17	0.18	0.76
Hmo (g/dL)	9.02	9.25	9.18	9.12	9.41	9.05	0.18	0.80	0.15
Hma (%)	27.06	27.75	27.56	27.38	28.25	27.17	0.54	0.81	0.14
Mcv (cells/µL)	135.17	135.25	142.93	154.19	128.12	121.43	11.16	0.07	0.09
Mch (g/dL)	47.44	46.34	47.20	51.36	42.73	39.86	3.39	0.01	0.19
Leu (cells/µL)	1617.12	1839.78	2145.01	1830.60	2191.41	1943.80	187.76	0.23	0.16
Mon, (cells/µL)	54.17	70.24	54.02	65.11	48.12	57.25	14.64	0.71	0.85
Eos (cells/µL)	32.23	45.86	33.96	30.32	48.31	47.29	18.20	0.25	0.78
H (cells/µL)	576.00	578.00	617.88	639.50	655.56	580.06	73.54	0.81	0.29
L (cells/µL)	1047.23	1084.53	1313.30	1114.54	1424.11	1261.43	137.63	0.11	0.29
H:L	0.54	0.52	0.50	0.58	0.46	0.47	0.07	0.24	0.87

P-values show the significance of the regression effects from orthogonal contrasts.
Ery, erythrocytes; Hmo, haemoglobin; Hma, haematocrit; Mcv, mean corpuscular volum; Mch, mean corpuscular haemoglobin; Leu, leukocytes; Mon, monocytes; Eos, eosinophils; H, heterophiles; L, lymphocytes; H:L, heterophiles:lymphocyte ratio.

Table 8. Mean values of the concentrations of corticosterone, triiodothyronine (T3) and thyroxine (T4) in the serum of broilers at 43 days of age.

Hormone (µg/dL)	Concentration of CrMet (mg/kg)						s.e.m.	*P*-value	
	0	0.10	0.20	0.40	0.80	1.20		Linear	Quadratic
Corticosterone	124.21	105.41	103.87	96.47	96.79	99.06	0.89	<0.01	<0.01
T3	46.15	57.25	61.21	56.86	58.27	56.52	3.16	0.23	0.04
T4	1.26	1.42	1.54	1.48	1.51	1.52	0.06	0.02	0.05
T3:T4	37.01	40.62	39.81	38.55	38.86	37.87	1.91	0.64	0.48

P-values show the significance of the regression effects from orthogonal contrasts.

Table 9. Equations from linear regression models (linear and quadratic polynomial regressions) and non-linear regression models (linear and quadratic broken-line) for the significative variables and their respective determination coefficients (R^2) and root mean squared error (RMSE).

Variable	Model	Equation of regression	R^2	RMSE	Min./max.	*P*-value
		Growth performance				
Bodyweight gain	QR	$y = 1514.2 + 165.68 \times x - 116.2 \times x^2$	0.25	51.4	0.71	0.01
Feed conversion ratio	QR	$y = 1.8745 - 0.2228 \times x + 0.163 \times x^2$	0.29	0.07	0.68	0.01
		Body temperatures				
CT 28 days	QR	$y = 41.8417 + 0.903 \times x - 0.7888 \times x^2$	0.24	0.31	0.572	0.03
MBT 28 days	LR	$y = 40.3853 - 0.2728 \times x$	0.35	0.37	–	0.04
		Haemogram				
Mch (g/dL)	LR	$y = 48.7643 - 6.4847 \times x$	0.17	10.67	–	0.01
		Hormones (µg/dL)				
Corticosterone	QR	$y = 117.7–67.7576 \times x + 44.7811 \times x^2$	0.72	5.39	0.757	<0.01
Corticosterone	LBL	$y = 97.4389 + 101.7 \times (0.2349 - x)$	0.81	4.43	0.235	<0.01
Corticosterone	QBL	$y = 97.857 + 296.3 \times (0.2919 - x)^2$	0.85	3.94	0.292	<0.01
T3	QR	$y = 51.3536 + 27.2631 \times x - 19.8608 \times x^2$	0.06	12.69	0.686	0.04
T3	LBL	$y = 58.2145 - 111 \times (0.1087 - x)$	0.12	12.32	0.109	<0.01
T3	QBL	$y = 58.2145 - 620.4 \times (0.1395 - x)^2$	0.12	12.32	0.140	<0.01
T4	LR	$y = 1.4213 + 0.1333 \times x$	0.07	0.25	–	0.02
T4	LBL	$y = 1.5098 - 1.5937 \times (0.1575 - x)$	0.14	0.24	0.158	<0.01
T4	QBL	$y = 1.5084 - 5.008 \times (0.2248 - x)^2$	0.14	0.24	0.225	<0.01

LR, linear regression model: $y = \beta_0 + \beta_1 \times (\beta_2 - X)$, where y is the response variable, X is the dietary CrMet concentration, β_0 is the intercept and β_1 is the linear coefficient of the regression; QR, quadratic polynomial regression model: $y = \beta_0 + \beta_1 \times X + \beta_2 \times X^2$, where y is the response variable, X is the dietary CrMet concentration, β_0 is the intercept, β_1 and β_2 are the linear and quadratic coefficients of the regression, respectively [maximum response concentration was obtained by: $-(\beta_1/2 \times \beta_2)$]; LBL, linear broken-line regression model: $y = \beta_0 + \beta_1 \times (\beta_2 - X)$, where $(\beta_2 - X) = 0$ for $X > \beta_2$, y is the response variable, X is the dietary CrMet concentration, β_0 is the value at the plateau, β_1 is the slope and β_2 is the dietary CrMet concentration at the break point; QBL, quadratic broken-line model: $y = \beta_0 + \beta_1 \times (\beta_2 - X)^2$, where $(\beta_2 - X) = 0$ for $X > \beta_2$, y is the response variable, X is the dietary CrMet concentration, β_0 is the value at the plateau, β_1 is the slope and β_2 is the dietary CrMet concentration at the break point. *P*-values show the significance of the linear regression effects from orthogonal contrasts when model is equal to LR or QR, and significance of the non-linear effects (SAS PROC NLIN) when model equal is to LBL or QBL.

R^2, determination coefficient for the regression equation; RMSE, root mean squared error; CT, cloacal temperature; MBT, mean body temperature; Mch, mean corpuscular haemoglobin; T3, triiodothyronine; T4, thyroxine.

There were significant ($P < 0.05$) effects of CrMet supplementation on the relative expression of HSP-70 in the breast of broilers at all supplement concentrations in comparison to the control diet (0 mg/kg de CrMet). The lowest HSP-70 expression was found with the inclusion level of 0.80 mg/kg CrMet. There were significant ($P < 0.05$) effects on the relative expression of IGF-1 in the breast of broilers supplemented with 0.80 and 1.20 mg/kg CrMet compared with those fed the control diet (0 mg/kg de CrMet). The highest IGF-1 expression was observed at the inclusion level of 0.80 mg/kg CrMet.

Discussion

Chromium is gaining recognition for its potential impact on the physiological variables of broiler chickens, resulting in enhanced performance (Hayat *et al.* 2020). In the current study, CrMet supplementation was found to improve BWG and FCR, with no significant effects on FI. These improvements may be attributed to its ability to enhance insulin action, thus promoting the utilisation of glucose and amino acids for growth (Sahin *et al.* 2010). Additionally, Cr is essential for proper glucose functioning and plays a crucial role in maintaining blood sugar concentrations (NRC 1997). Acting as the active component of chromodulin, it further enhances insulin signalling, potentially affecting carbohydrate metabolism (Vincent 2001; Almeida *et al.* 2010; Valente Junior *et al.* 2021). Furthermore, Cr facilitates the uptake of amino acids into tissues, resulting in more efficient muscle building (Anderson and Allen 1994). Previous studies have reported the beneficial effects of CrMet supplementation on broiler performance (Zheng *et al.* 2016; Arif *et al.* 2019; Dalólio *et al.* 2021; Youssef *et al.* 2022). However, it is important to note that Cr effects on growth performance can vary owing to factors such as different stress conditions, basal diets, nutrient content, and variations in Cr source (Ghazi *et al.* 2012). In the present study, the inclusions of 0.71 and 0.68 mg of CrMet were estimated to optimise BWG and FCR respectively.

Among body-temperature measurements in broilers, measuring cloacal temperature is crucial because it reflects

Table 10. Mean relative expression values for the heat-shock protein gene-70 (HSP-70) and insulin-like growth factor (IGF-1) in the breast of 43-day-old broilers.

Comparison among CrMet concentrations (mg/kg)	HSP-70		IGF-1	
	Fold change	*P*-value	Fold change	*P*-value
0 vs 0.10	4.72	<0.01*	2.23	0.09
0 vs 0.20	4.38	<0.01*	2.12	0.11
0 vs 0.40	3.94	<0.01*	2.07	0.12
0 vs 0.80	2.39	0.03*	2.82	0.03*
0 vs 1.20	2.57	0.02*	2.77	0.03*
0.10 vs 0.20	−1.07	0.84	−1.05	0.91
0.10 vs 0.40	−1.20	0.64	−1.08	0.86
0.10 vs 0.80	−1.97	0.09	1.26	0.61
0.10 vs 1.20	−1.83	0.13	1.24	0.64
0.20 vs 0.40	−1.11	0.78	−1.03	0.95
0.20 vs 0.80	−1.83	0.13	1.32	0.54
0.20 vs 1.20	−1.70	0.19	1.30	0.56
0.40 vs 0.80	−1.64	0.21	1.36	0.50
0.40 vs 1.20	−1.52	0.29	1.34	0.52
0.80 vs 1.20	−1.08	0.84	−1.02	0.96

*Significant ($P < 0.05$).

the core body temperature (Phalen *et al.* 1996). Notably, Giloh *et al.* (2012) found that the cloacal temperature tends to remain stable regardless of external environmental conditions. However, Altan *et al.* (2003) observed that broilers exposed to HS might experience an increase in cloacal temperature. In the current study, cloacal temperature variation was observed, ranging from 40.81°C to 42.15°C at Day 28 and from 42.24°C to 42.63°C at Day 42. These findings align with previous research by Ryu *et al.* (2016), which reported a cloacal temperature range from 41°C to 42°C in adult birds. The linear increase in cloacal temperature at Day 28 with an increasing supplementation of CrMet could be attributed to an increase in metabolic rate, affecting the hormone concentrations. In contrast, Norain *et al.* (2013) noted a reduction in cloacal temperature and respiratory rate of broilers exposed to HS and fed diets containing 2.00 mg/kg of chromium chloride ($CrCl_3$). Regarding the linear reduction in mean body temperature at Day 28 with increasing CrMet concentrations in the broiler diets, this might indicate that, despite the increase in cloacal temperature, birds exhibited a higher efficiency in heat exchange with the environment. This phenomenon is primarily observed in body parts not covered by feathers, such as feet, comb, and wattle, which are variables included in the mean body-temperature index. Although mean skin temperature is calculated on the basis of cloacal temperature and mean body temperature, no variations were observed in this parameter at Day 28. The lack of response to body temperature indices at Day 42 could be an indication of the adaptability of broilers to HS.

Heat stress induces immunosuppression in broilers, and the change in the weight of lymphoid organs (bursa, thymus, and spleen) is an indirect measure used to assess the condition of the immune system (Lu *et al.* 2019). In the present study, the supplementation of CrMet did not have a significant effect on the weight of lymphoid organs. Similar results were observed by Silva *et al.* (2014) for broilers fed diets supplemented with CrMet and reared under HS. However, Ghazi *et al.* (2012) observed an increase in the relative weight of the thymus and spleen in broilers under HS when they were supplemented with 1.20 mg/kg of CrMet and $CrCl_3$. The variations in results across different studies may be attributed to several factors, including differences in facility environment and hygiene, local sanitary challenges, the severity of the stressor (heat or cold), dietary composition (such as the inclusion of exogenous enzymes, particularly phytase, electrolyte balance, and the use of synthetic amino acids), as well as the statistical models used for analysis (Dalólio *et al.* 2021).

The blood profile is particularly sensitive to temperature changes and may be a potential indicator of physiological responses of broilers exposed to HS (Altan *et al.* 2003). Quantitative and morphological changes in the blood cells of broilers are linked to HS, particularly affecting parameters such as hematocrit values, number of circulating leukocytes and erythrocytes, and the haemoglobin content in erythrocytes (Borges *et al.* 2003). In the present study, only the mean corpuscular haemoglobin content was altered as a result of CrMet supplementation. The mean corpuscular haemoglobin and mean corpuscular volume are indices used to measure the amount of haemoglobin per erythrocyte. These indices provide insights into the presence of anemia status and bone marrow's ability to produce red blood cells of normal size and metabolic capacity (Campbell 1995).

Vincent (2010) proposed a possible explanation that Cr may compete with iron (Fe) for the same absorption site and transportation through the transferrin, potentially resulting in lower free circulating Fe and inducing anemia. However, in the current study, Cr was provided in a complex with methionine (Met), which is absorbed via active dependent co-transportation with sodium, thus Cr attached to Met would be absorbed together. The increase in the mean corpuscular haemoglobin observed in the present study, even in the context of possible anemia, should theoretically influence performance because the red blood cells are responsible for transporting oxygen and nutrients to the cells. However, this effect was not observed. In this way, to better understand the relationship between Cr supplementation, red blood-cell morphology, and the inflammatory response linked to protein content and concentrations in the blood of broilers, more research is needed. Regarding the inflammatory response in broilers under HS, Roll *et al.* (2010) emphasised the importance of the H:L ratio as a parameter for evaluating the immune process. Ebrahimzadeh *et al.* (2012) observed effects on the concentration of heterophiles and decrease in

H:L in broilers exposed to HS and fed a diet containing 0.80 mg/kg of CrMet, suggesting an improvement in the immune response. However, in this study, the supplementation of CrMet did not significantly affect the H:L.

The supplementation of CrMet decreased circulating COR concentration, which was efficient in mitigating the deleterious effects of HS. Heat stress triggers the production of pro-inflammatory cytokines, such as interleukin 6, C-reactive protein and tumor necrosis-factor alpha (TNF-α), which in turn stimulate the hypothalamus to secrete corticotropic hormone that acts on the adrenal gland producing COR in broilers (Sahin *et al.* 2010; Ghazi *et al.* 2012). Increased concentrations of COR cause excess free radicals and suppress the proliferation of interleukin-2 (Siegel 1995; Rao *et al.* 2016), impairing immune response and performance.

According to Orhan *et al.* (2012) and Akdemir *et al.* (2015), Cr increases hepatic expression of protein IκBα and decreases free radical production and tumor necrosis factor-Kb in meat-type quails under HS. That results in reduction of transcription of genes forming apoptotic factors, which decreases proteolysis and lipid peroxidation in quails under HS. Rao *et al.* (2012, 2016) observed that supplementation with CrMet and Cr-yeast in broilers increased glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase activity, which decreased plasma lipid peroxidation in broiler chickens raised under thermal comfort temperature and HS. Li *et al.* (2018) reported that supplementation of 0.20 mg/kg of CrPic increased superoxide dismutase and reduced malondialdehyde concentrations in heat-stressed ducks. Those results indicated that Cr can reduce the oxidative stress caused to the cells when birds are exposed to HS.

Ebrahimzadeh *et al.* (2012) observed that CrMet at all levels of inclusion studied decreased cortisol concentration of broiler chickens under HS. Bahrami *et al.* (2012) evaluated CrMet and $CrCl_3$ supplementation in broilers raised in HS and observed a reduction of cortisol concentration at 42 days, with a more pronounced effect for the CrMet source. Contrarily, Jahanian and Rasouli (2015) observed that 1.00 mg/kg CrMet supplemented in diets of broilers under HS was not sufficient to reduce plasma COR and to restores lymphoid organ weights in comparison to broilers raised under thermal-comfort temperature.

In addition to reducing COR concentrations, Sahin *et al.* (2002) also observed an increase in T3 and T4 concentrations in broilers exposed to HS and fed diets supplemented with CrPic during the period from 21 to 42 days. Triiodothyronine is the active form of the hormone, whereas T4 serves as a reservoir for T3. Both hormones stimulate basal metabolism in animals and have a trophic effect on the intestinal mucosa (Daher *et al.* 2009; Martinez and Ortiz 2017). This, in turn, favoured an increase in apparent metabolisable energy, indicating a higher retention of nitrogen for growth in relation to gross energy of the diet. Consequently, this improvement in performance aligns with the findings from our research conducted by Dalólio *et al.* (2021). The precise mechanism through which Cr leads to an increase in T3 and T4 concentrations is not yet well elucidated. It could be related to indirect changes that enhance cellular sensitivity to insulin recognition, because it has been reported that insulin can affect the mechanisms of insulin resistance as well as T3 and T4 concentrations (Godini *et al.* 2015).

In HS conditions, the expression levels of HSP tend to increase, especially in fast-growing broilers (Cedraz *et al.* 2017). This increase in HSP occurs to assist the process of synthesis and maturation of new proteins, particularly in cells that have been damaged during exposure to HS (Gupta *et al.* 2007).

Reducing HSP-70 expression levels contributes to the reduction of oxidative stress in the breast of broilers. This is particularly significant in broilers with a high protein content, because they have elevated activity and heat production rates. Rajkumar *et al.* (2018) observed similar results when they evaluated the supplementation of 2.00 mg/kg of Cr-yeast, which led to a reduction in HSP-70 expression in the muscle, liver, heart, and spleen of broilers raised under HS compared with a control group that received 0 mg/kg of Cr-yeast. Ezzat *et al.* (2017) evaluated the supplementation of 1.20 mg/kg of CrPic and observed a reduction in HSP-70 in liver of broilers reared under HS in relation to control diet (0 mg/kg of CrPic). Akdemir *et al.* (2015) evaluated two levels of Cr-histidinate supplementation (0.40 and 0.80 mg/kg) in the diet of meat-type quails exposed to HS and observed a remarkable reduction of 29% in liver HSP-70 expression. Thus, supplementation of CrMet in the diet of modern fast-growing broiler strains is a viable alternative to mitigate oxidative stress in the breast of broilers, which may improve meat quality and yield.

Heat stress has also been reported to decrease IGF-1 expression and to accelerate the ubiquitin-proteasome metabolic pathways, resulting in proteolysis and increased metabolism. Decreased IGF-1 expression under conditions of oxidative stress can lead to proteolysis, diverting essential amino acids away from growth and towards other metabolic processes (Del Vesco *et al.* 2013). Although the liver is the primary organ responsible for IGF-1 expression in broilers under HS (Del Vesco *et al.* 2015), the breast muscle is also capable of considerable IGF-1 expression, significantly contributing for protein synthesis (Vignale *et al.* 2017; Wen *et al.* 2017). IGF-1 acts as a mediator of growth hormone (GH) and is directly linked to protein synthesis (Cruzat *et al.* 2008). Therefore, increased IGF-1 expression signifies that amino acids are being directed towards protein deposition and indicates a reduction in free radical production. Glucocorticoids, such as corticosterone, have an antagonistic effect on insulin action, whereas thyroid hormone (T3) is synergistic with insulin and tolerant to GH (Macari and Maiorka 2017). These factors collectively explain the beneficial effects of Cr on broiler metabolism, including increased insulin production and a reduction in plasma corticosterone concentration.

Heat stress has been shown to reduce the plasma concentration of Cr and its deposition in tissues, while increasing urinary excretion (Vincent 2000; 2010), which can lead to a slight deficiency in animals. In such case, the ability of LMWCr to enhance insulin recognition by target cells may be impaired. Therefore, the supplementation of Cr for broilers can promote physiological modifications in endocrine and metabolic pathways, as well as alterations in the expression of specific genes to promote endogenous heat dissipation to the exterior, all without compromising the development of broilers.

Overall, supplementation with CrMet has been found to promote various beneficial changes in broiler chickens. These changes include a reduction in body temperature, serum corticosterone and HSP-70 expression in muscle. Furthermore, an increase in serum concentrations of T3 and T4 was observed, along with a corresponding increase in muscle IGF-1 concentrations. These physiological changes have a positive impact on broiler performance, as was observed in the findings by the same authors (Dalólio *et al*. 2021), which are summarised in Table 8.

In conclusion, supplementation with 0.80 mg/kg is recommended to enhance broiler performance, optimise hormonal profile, reduce HSP-70 expression, and increase IGF-1 concentrations in the breast tissue of broilers exposed to HS during the period from 22 to 43 days of age.

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