The effect of total sulfur amino acid levels on growth performance and bone metabolism in pullets under heat stress

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ABSTRACT A study was conducted to investigate the effects of total sulfur amino acid (**TSAA**) levels on performance and bone metabolism in pullets under heat stress (**HS**). Hy-Line W36 day-old pullets ($n = 216$) were randomly distributed in 3 dietary treatments (70, 85, and 100% of TSAA), with 6 replicates of 12 birds. The treatments were defined as percentages of the TSAA level recommendation (100, 85, and 70%), and 85 and 100% of TSAA were obtained by adding L-Methionine to the basal deficient diet (70% of TSAA). The birds were raised under HS (35°C/7 h/D) from 1 to 18 wk. At 6, 12, and 18 wk, growth performance was measured. At 12 and 18 wk, bone weight, ash, collagenous (**ColP**), and noncollagenous proteins (**NColP**), tissue volume (**TV**), bone mineral content (**BMC**), and mineral density from total, cortical, and trabecular bones were evaluated. The means were subjected to ANOVA and, when significant ($P \leq 0.05$), were compared by Dunnett's test. Regression analyses were performed to evaluate trends of TSAA dose response. Overall, birds fed 70% of TSAA showed poor growth and feed efficiency compared with other groups. Additionally, in at least 1 phase, birds fed 70% of TSAA showed lower bone ash, NColP, total BMC, and TV and higher ColP than the other treatments, whereas the cortical and trabecular TV and BMC were lower than 100% of TSAA ($P < 0.04$). Quadratic effects of TSAA levels on body weight gain (**BWG**) were found, and the level for maximum BWG was 95% of the TSAA recommendation ($P < 0.03$, $R^2 > 0.83$). In conclusion, the use of a TSAA-deficient diet resulted in poor performance and delayed bone development. Additionally, the use of 100% of TSAA led to better initial structural bone development than 85% of TSAA. Therefore, the TSAA level recommended by the primary breeder guideline was enough to support growth and bone quality under HS, suggesting that HS does not alter TSAA requirement in pullets.

Key words: bone metabolism, heat stress, pullets, total sulfur amino acids

2020 Poultry Science 99:5783–5791
https://doi.org/10.1016/j.psj.2020.06.081

INTRODUCTION

Methionine (**Met**) and Cystine (**Cys**), the total sulfur amino acids (**TSAA**), are essential for poultry. They have been shown to participate in protein deposition, polyamine synthesis, and as part of the antioxidant system through glutathione and taurine metabolism (Stipanuk, 2004; Bunchasak, 2009). Methionine can be converted to Cys in an irreversible reaction catalyzed by the enzymes cystathionine β-synthase and cystathionine-γ-lyase in the transulfuration pathway (Métayer et al., 2008). Therefore, the Met supplementation can satisfy both Met and Cys requirements.

The sexual maturity and production performance of laying hens are directly dependent on their body development during the first 18 wks of life (D'Agostini et al., 2017). Furthermore, structural bones are formed during the pullet phase, whereas after the onset of egg production, there is a shift in bone development toward medullary bone formation. Because higher Ca levels are required for eggshell synthesis during the egg production period, bone is resorbed not only from the medullary but also from trabecular and cortical portion (Whitehead, 2004; Narvaez-Solarte et al., 2006). As the resorption process continues throughout the whole egg production period, there are increased bone loss and higher risk of osteoporosis in laying hens, which is an important economic and welfare issue (Whitehead and Fleming, 2000).

The influence of TSAA on the bone quality of pullets and laying hens is not well established, even though there are some available data in the literature concerning other species. Studies have shown that Met restriction could reduce collagen formation and bone differentiation

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Received February 24, 2020.
Accepted June 19, 2020.

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in mice (Ouattara et al., 2016), whereas Met supplementation was shown to improve cartilage health in humans (Blewett, 2008). Moreover, lower plasmatic Cys levels were shown to reduce the bone mineral density of humans, possibly by reducing collagen formation (Baines et al., 2007).

Another economic and welfare problem faced in animal production is heat stress (**HS**). Heat stress ultimately leads to cellular oxidative stress in poultry, and the TSAA could mitigate its negative effects by increasing glutathione production, thus improving the antioxidant capacity (Bunchasak and Silapasorn, 2005; Del Vesco et al., 2015). Additionally, during HS, a reduction in crude protein and Met digestibility has been reported (Wallis and Balnave, 1984; Zuprizal et al., 1993; Attia et al., 2016).

Therefore, the TSAA could be used as a dietary tool to maximize the bone growth and mineral deposition and minimize the negative effects of HS in modern pullets. For this reason, the objective of this study was to evaluate the effects of different TSAA levels by adding L-Methionine (**L-Met**) on growth performance and bone quality in Hy-Line W36 pullets raised under HS.

MATERIALS AND METHODS

General Procedures

The experiment was conducted under the approval of the Institutional Animal Care and Use Committee of University of Georgia (Athens, Georgia). A total of two hundred sixteen 1-day-old HyLine W36 pullet chicks were distributed in a completely randomized design with 3 dietary treatments and 6 replicates of 12 birds each. The pullets were allocated to 18 identical cages (88 cm length x 47 cm width x 39 cm height) equipped with drinkers and feeder, providing free access to water and feed until 18 wk of age. The lighting program followed the Hy-Line W36 (2016) guide, and the light period was decreased in 1 h weekly from wk 1 (20h light/D) until wk 11 (12h light/D), and after this week on, it was kept at 12h light/D until 18 wk of age. Room temperature (°C) setup followed by the recommended by the line guide until 2 wk of age, and the pullets were subjected to chronic cyclic HS from 2 to 18 wk. The room temperature was set to be 35°C/7 h/D, followed by 17h of the temperature recommended by HyLine W36 guideline according to the age (Hy-Line W36, 2016). The room temperature was increased at 10 AM and decreased at 5 PM, daily and manually, whereas the relative humidity (**RH**, %) was not controlled in the room. The temperature and RH were recorded hourly by 2 data loggers (**HOBO**) and summarized by the Onset HOBOware (Software for HOBO Data Loggers and Devices, 2002-2017, version 3.7.13, Onset Computer Corporation).

Experimental Diets and Data Collection

The diets were based on corn and soybean meal and formulated to reach the HyLine W36 nutrient specifications for each phase, except for Met and TSAA levels. The experimental period was divided into starter 1 (1 to 3 wk), starter 2 (4 to 6 wk), grower (7 to 12 wk), developer (13 to 15 wk), and prelay (15 to 18 wk) phases (Hy-Line W36, 2016) (Table 1). The TSAA levels were defined as a percentage (70, 85, and 100%) of the total TSAA levels recommended by the line guideline, which are 0.83, 0.83, 0.75, 0.67, and 0.74%, for starter 1, starter 2, grower, developer, and prelay phases, respectively. The TSAA dietary levels were obtained by adding L-Met (CJ CheilJedang, Seoul, Korea) to the basal diet (70% of TSAA without Met supplementation) as a replacement of the inert component (Solka flock) until we reached the levels of 85 and 100% of TSAA. The supplementation levels of L-Met to reach each one of those levels were 0.13, 0.13, 0.11, 0.10, and 0.11% of L-Met for 85% of TSAA and 0.25, 0.25, 0.22, 0.20, and 0.22% of L-Met for 100% of TSAA, for starter 1, starter 2, grower, developer, and prelay phases, respectively. The crude protein level was kept constant for all diets using glutamine to balance the addition of L-Met.

The birds and feed were collectively weighed by cage, at 6, 12, and 18 wk of the experiment, to determine body weight gain (**BWG**), feed intake (**FI**), and feed conversion ratio (**FCR**). Mortality was recorded daily. At wk 12 and 18, 1 bird per replicate was selected from a range of ±10% the cage average body weight and euthanized by cervical dislocation. Both tibiotarsi and femora were collected for bone ash, collagenous, and noncollagenous protein (**ColP** and **NColP**, respectively), and microcomputed tomography (**MicroCT**) analyses. The body temperature was measured, weekly, from 10 randomly selected birds. A digital clinical thermometer was partially inserted into the cloaca and kept in direct contact with the mucosa until the reading became stable. The body temperature (**MicroCT**) was measured before the increase in room temperature (9 AM) and during high room temperature (3 PM), and these values were compared and used as a parameter for assessing HS.

Bone Quality

Bone Ash and Collagenous and Noncollagenous Protein For ash content determination, the Method 932.16 from AOAC International (1990) was used. The right tibiotarsi were cleaned of any adhering tissue and dried at 100°C for 24h. After drying, fat from the bone was extracted in a Soxhlet apparatus for 48h using Hexane (Fisher Scientific International Inc., Waltham, MA) as a solvent. Then, the fat-free bones were dried in an oven at 100°C for 24 h. The dry-fat free weight was recorded, and the bones were ashed at 600°C overnight, cooled in a desiccator, and weighed.

The ColP and NColP determination followed a methodology adapted from (Barbosa et al., 2010) and previously described by Castro et al. (2019). Briefly, left tibiotarsi were cut longitudinally, and the bone marrow was washed out. Next, the fat was extracted from the bones using the same methodology for ash analysis, and dry fat-free bones were continuously demineralized

Table 1. Basal diet formulation for all phases (1 to 18 wk, as-fed basis; % diet).

Ingredients	Starter 1 (1–3 wk)	Starter 2 (4–6 wk)	Grower (7–12 wk)	Developer (13–15 wk)	Prelay (15–18 wk)
Corn	68.59	70.00	72.00	73.00	70.52
Soybean meal (48%)	23.96	23.60	19.48	14.91	15.34
Soybean oil	1.00	1.01	1.12	2.64	1.50
Limestone	0.67	0.72	0.81	1.94	4.69
Deflourinated phosphate	2.08	2.02	1.95	1.90	2.07
Solka floc	0.45	0.98	1.99	2.40	2.06
Salt	0.30	0.30	0.30	0.30	0.30
Vitamin mix[1]	0.50	0.50	0.50	0.50	0.50
Mineral mix[2]	0.08	0.08	0.08	0.08	0.08
L-Methionine	-	-	-	-	-
Cystine	-	-	-	-	0.05
L-lysine	0.35	0.25	0.26	0.27	0.29
Threonine	0.18	0.13	0.13	0.13	0.15
Arginine	0.12	0.04	0.06	0.07	0.10
Isoleucine	0.08	0.06	0.08	0.08	0.14
Tryptophan	-	-	0.01	0.03	0.05
Valine	0.02	-	0.04	0.05	0.11
Glutamine	1.57	0.26	1.14	1.65	2.00
Coban 90	0.05	0.05	0.05	0.05	0.05
ME (kcal/kg)	3,030	3,030	3,030	3,100	2,940
CP (%)	20.00 (18.67)[3]	18.25 (17.50)	17.50 (15.84)	16.00 (15.70)	16.50 (15.78)
Lysine (%)	1.15	1.07	0.96	0.83	0.85
Methionine (%)	0.28 (0.37)	0.28 (0.36)	0.26 (0.34)	0.23 (0.29)	0.23 (0.30)
Cystine (%)	0.30 (0.31)	0.30 (0.33)	0.27 (0.29)	0.24 (0.24)	0.29 (0.28)
Met + Cys (TSAA, %)	0.58 (0.68)	0.58 (0.69)	0.53 (0.63)	0.47 (0.53)	0.52 (0.58)
Ca (%)	1.00	1.00	1.00	1.40	2.50
Available P (%)	0.50	0.49	0.47	0.45	0.48

Abbreviation: TSAA, total sulfur amino acid.

[1] Provided per kg of DSM Vitamin premix: Vit. A 2,204,586 IU, Vit. D_3 200,000 ICU, Vit. E 2,000 IU, Vit. B12 2 mg, Biotin 20 mg, Menadione 200 mg, Thiamine 400 mg, Riboflavin 800 mg, d-Pantothenic Acid 2,000 mg, Vit. B6 400 mg, Niacin 8,000 mg, Folic Acid 100 mg, Choline 34,720 mg.

[2] Provided per kg of Mineral premix: Ca 0.72 g, Mn 3.04 g, Zn 2.43 g, Mg 0.61 g, Fe 0.59 g, Cu 22.68 g, I 22.68 g, Se 9.07 g.

[3] Analyzed values.

by a solution of ethylenediamine tetraacetic acid for the extraction of NColP. The NColP solution was collected, and protein was quantified using Bradford's method and bovine serum albumin 2 mg/µl as a standard (Bradford, 1976). For ColP, the ethylenediamine tetraacetic acid residue was washed off from the bones, which were then dried, weighed, and ground, and N was analyzed by combustion method (LECO) (Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia). The ColP was determined by multiplying N x 6.25. The NColP and ColP results were given in absolute weight and as a percentage of fat-free dry bone weight without bone marrow.

Microcomputed Tomography For MicroCT analysis, the sample preparation and analysis followed the methodology described by Castro et al. (2019). The right femora were scanned using Skyscan X-ray Microtomography (Bruker Corporation, Billerica, MA), with

Table 2. Means of body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR) and abdominal fat (Fat) according to TSAA levels.

		TSAA levels (%)[1]			*P*-value				
Age (wk)	Traits	70%	85%	100%	ANOVA	Regression[2]	Maximum response (%)	R^2	SE
1–6	BWG (g)	289.98	333.81[3]	341.00[3]	<0.0001	0.0013^{Q}	94.46	0.87	5.8413
	FI (g/bird/d)	19.28	19.44	19.32	NS	NS	-	-	0.1000
	TSAA intake (mg/bird/d)	132.00	155.50[3]	180.67[3]	<0.0001	$<0.0001^{L}$	-	0.96	0.0048
	FCR (g/g)	2.80	2.45[3]	2.38[3]	<0.0001	$<0.0001^{Q}$	99.08	0.93	0.0456
7–12	BWG (g)	387.42	460.44[3]	470.12[3]	<0.0001	0.0030^{Q}	94.77	0.83	9.7944
	FI (g/bird/d)	48.15	49.92	48.60	NS	NS	-	-	0.4912
	TSAA intake (mg/bird/d)	303.50	359.67[3]	355.00[3]	<0.0001	$<0.0001^{L}$	-	0.53	0.0069
	FCR (g/g)	4.35	3.80[3]	3.62[3]	<0.0001	0.0359^{Q}	104.18	0.81	0.0840
13–18	BWG (g)	422.26	451.33[3]	447.98[3]	0.0273	0.0266^{L}	-	0.25	5.1014
	FI (g/bird/d)	64.22	67.14	64.97	NS	0.0243^{Q}	86.15	0.31	0.5407
	TSAA intake (mg/bird/d)	356.33	460.00[3]	471.00[3]	<0.0001	0.0001^{Q}	96.75	0.95	0.0128
	FCR (g/g)	5.32	5.21	5.08	NS	0.0408^{L}	-	0.25	0.0477

Abbreviations: BWG, body weight gain; FCR, feed conversion ratio; FI, feed intake; NS, Not significant; TSAA, total sulfur amino acid.

N = 6.

[1] 70, 85, and 100% of TSAA are correspondent to: 0.58/0.71/0.83% from 1 to 6 wk; 0.53/0.64/0.75% from 7 to 12 wk; 0.47;0.52/0.57;0.63/0.67;0.74% from 13 to 18 wk, respectively.

[2] Linear (L) or Quadratic (Q).

[3] Means differ from 70% of TSAA by Dunnett test ($P \leq 0.05$).

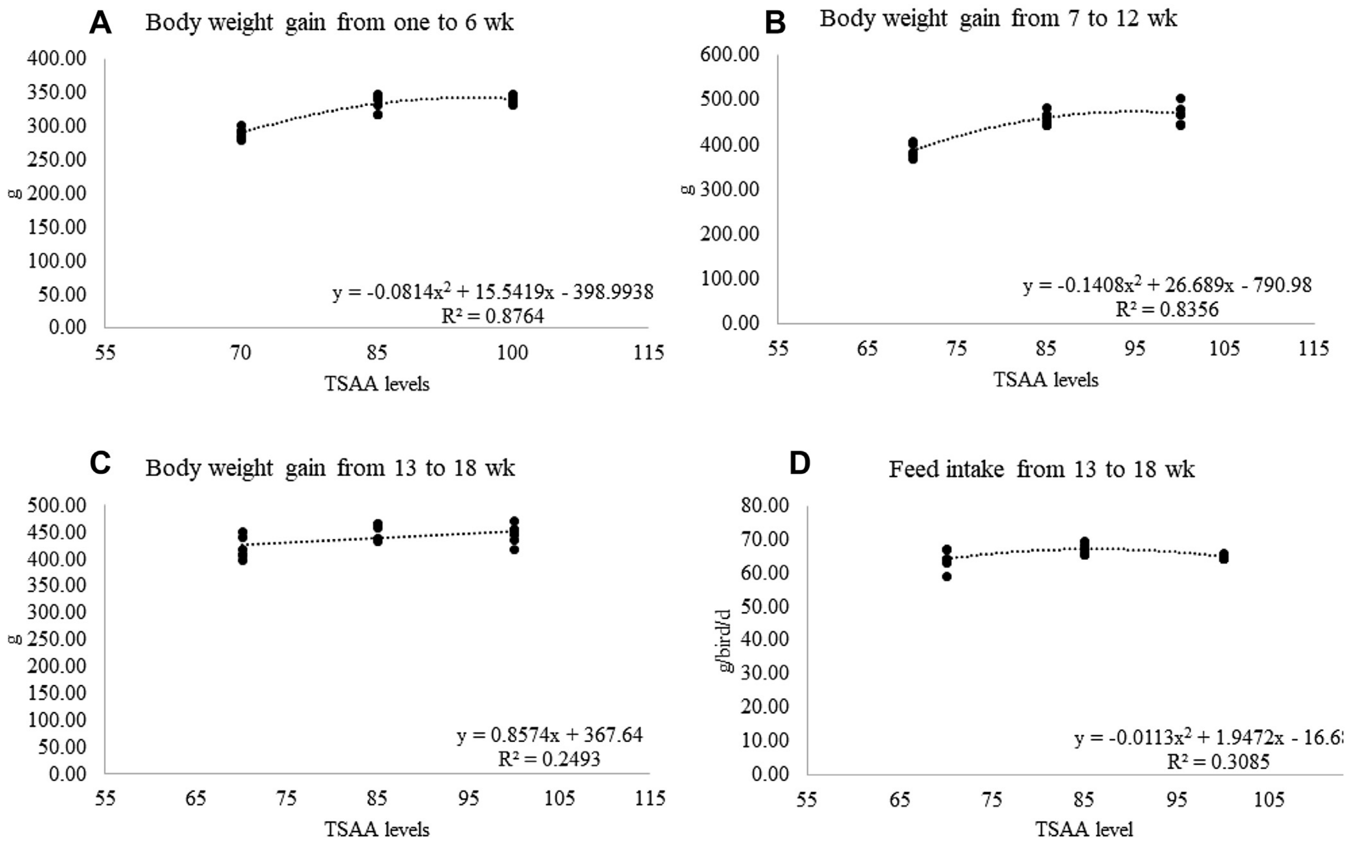

Figure 1. Body weight gain from 1 to 6 (A), 7 to 12 (B), and 13 to 18 (C) wk, and feed intake from 13 to 18 wk (D) according to TSAA levels. Abbreviation: TSAA, total sulfur amino acid.

high resolution, a magnification of 26.6 mm (pixel size), and aluminum filter (0.1 mm). The scanning settings were as follows: voltage 70 kV/current 142 uA for wk 12 and voltage 82 kV/current 121 uA for wk 18. Reconstruction of projection images was performed by NRecon Software to obtain the cross-sectional image data set, and these images were adjusted by using DataViewer Software (Bruker Corporation). The volume of interest consisted of 200 slices (5.322 mm), selected from the distal supracondylar area of the bone. The volume of interest was then analyzed, and total bone, cortical, and trabecular tissue volume (**TV**), bone mineral content (**BMC**), and bone mineral density (**BMD**) were calculated by using 8 mm phantoms (0.25 and 0.75 g cm^{-3} CaHA) as the density reference (CTAn Software, Bunker Corporation).

Statistical Analysis

A test for homogeneity of variances and normality of studentized residuals was performed, followed by one-way ANOVA. When significant, Dunnett's t-test was used as a *post hoc* test to compare each one of the treatments against 70% of TSAA. Additionally, a polynomial regression was performed for each variable against the dietary TSAA levels to determine a linear or quadratic trend. When a quadratic effect was found, the maximum and minimum responses were obtained by $\beta_1/(2\times\beta_2)$, in which β_1 is the linear coefficient, and β_2 is the quadratic coefficient of the equation. All statistical procedures were performed using the Proc GLM procedure from SAS University Edition (SAS Institute, 2017), and the statements of significance were based on $P \leq 0.05$.

RESULTS

The BWG of pullets fed 85 and 100% of TSAA was higher than the one of birds fed 70% of TSAA for all the analyzed phases ($P < 0.027$) (Table 2). There were quadratic effects of TSAA levels on this trait from 1 to 6 ($P = 0.001$, $R^2 = 0.87$) to 7 to 12 ($P = 0.003$, $R^2 = 0.83$) wk of age. The calculated TSAA levels for maximum BWG were 0.79% (95.46% of the HyLine W36 TSAA requirement) during 1 to 6 wk and 0.71% of TSAA (94.77%) during 7 to 12 wk (Figure 1A and 1B). A linear effect of TSAA levels on BWG was observed at 13 to 18 wk of age ($P = 0.026$, $R^2 = 0.25$), in which increasing levels of TSAA led to increased BWG (Figure 1C). For FI, the treatments did not differ from the 70% of TSAA fed group in any phase ($P > 0.05$). However, there was a quadratic effect of TSAA on FI at 13 to 18 wk of age ($P = 0.0243$, $R^2 = 0.31$). The calculated TSAA level which resulted in maximum FI was 0.61% of TSAA (86.15% of the HyLine W36 TSAA requirement) (Figure 1D).

The TSAA intake was lower for birds fed 70% of TSAA compared with 85 and 100% during all the phases ($P < 0.001$). Linear effects of TSAA levels on TSAA

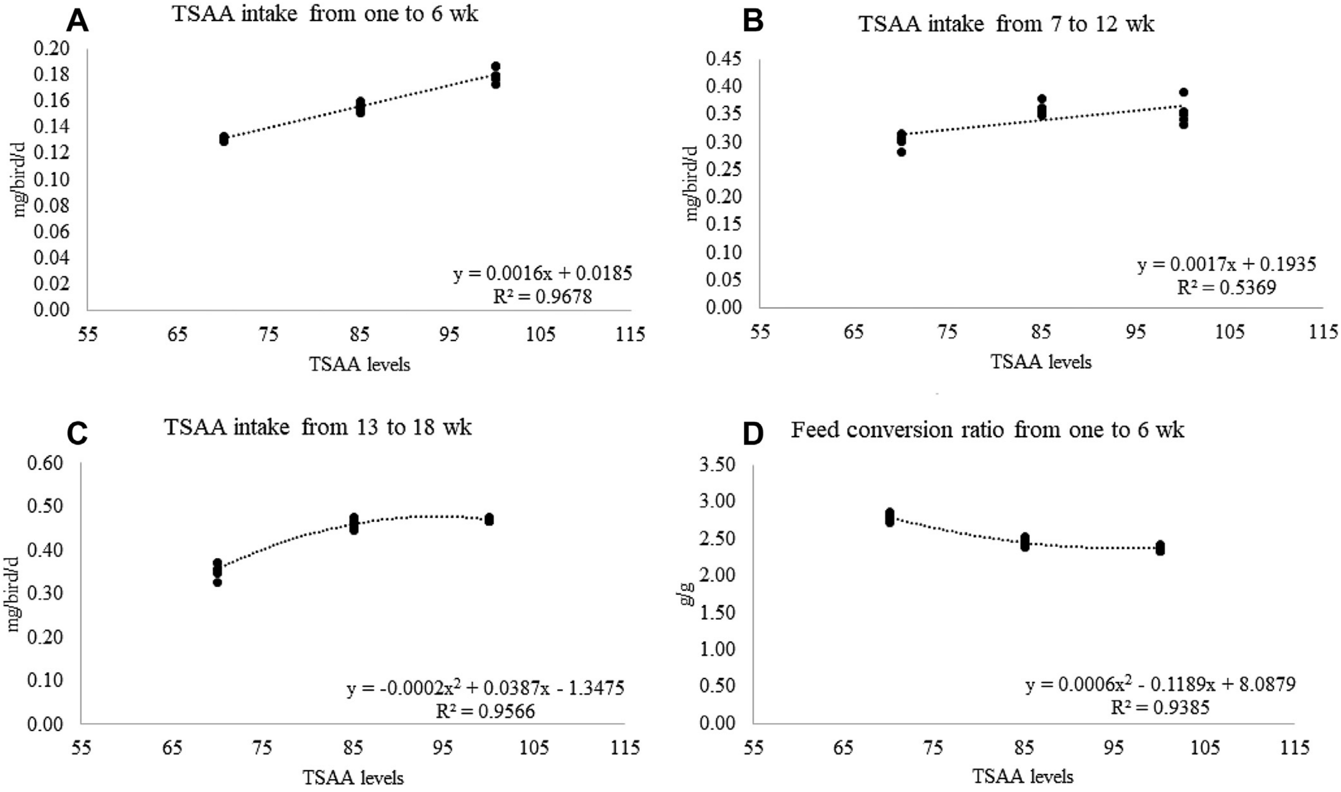

Figure 2. TSAA intake from 1 to 6 (A), 7 to 12 (B), and 13 to 18 wk (C), and feed conversion ratio from 1 to 6 wk (D) according to TSAA levels. Abbreviation: TSAA, total sulfur amino acid.

intake were observed during 1 to 6 wk ($P < 0.001$, $R^2 = 0.96$) and 7 to 13 wk ($P < 0.001$, $R^2 = 0.53$), in which increasing levels of TSAA resulted in increasing TSAA intake (Figures 2A and 2B). A quadratic effect of TSAA levels on TSAA intake was observed during 13 to 18 wk ($P < 0.001$, $R^2 = 0.95$), in which the calculated level for maximum TSAA intake was 0.68% of TSAA (96.75% of the HyLine W36 TSAA requirement) (Figure 2C). The FCR was lower for birds fed 85 and 100% of TSAA compared with birds fed 70% of TSAA during 1 and 6 and 7 and 12 wk periods ($P < 0.001$). Additionally, there were quadratic effects of TSAA levels on FCR for these same phases ($P < 0.001$, $R^2 = 0.93$ from 1-6 wk, and $P = 0.035$, $R^2 = 0.81$ from 7–13 wk). The calculated TSAA levels for minimum FCR were 0.82% of TSAA (99.08% of the HyLine W36 TSAA requirement) during 1 to 6 wk, and 0.78% (104.18%) during 7 to 12 wk (Figures 2D and 3A). Additionally, a linear effect of TSAA levels on FCR was observed from 13 to 18 wk ($P = 0.040$, $R^2 = 0.25$), and increasing levels of TSAA led to decreased FCR (Figure 3B).

For bone quality evaluation, at 12 wk of age, the ColP (%) was higher when birds were fed 70% compared with 85 and 100% TSAA ($P = 0.001$), whereas the ColP (g) was not significantly different ($P > 0.05$) (Table 3). The NColP (%) was lower when birds were fed 85% compared to 70% of TSAA ($P = 0.049$), and the NColP (mg) was higher when birds were fed 100% of TSAA compared with 70% of TSAA ($P = 0.027$). The bone ash, total bone TV, and total BMC were higher for both 85 and 100% compared with 70% of TSAA ($P < 0.005$). Additionally, the cortical bone TV, cortical BMC, trabecular TV, and trabecular BMC were higher for birds fed 100% of TSAA compared with the ones fed 70% of TSAA ($P < 0.044$). No linear or quadratic effects of TSAA levels on these traits were observed during this phase ($P > 0.05$).

At wk 18, bone weight and ash were higher for birds fed 85 and 100% of TSAA compared with the negative control group (70% of TSAA) ($P < 0.048$) (Table 4). The total bone TV, cortical TV, and cortical BMC were higher for birds fed 85% of TSAA compared with those fed 70% of TSAA ($P < 0.046$). Quadratic effects of TSAA levels on cortical TV ($P = 0.018$, $R^2 = 0.50$) and cortical BMC ($P = 0.011$, $R^2 = 0.51$) were found at this age. The calculated level for maximum cortical TV was 88.59% of the HyLine W36 TSAA requirement, whereas the calculated level for maximum cortical BMC was 87.94% of the HyLine W36 TSAA requirement (Figures 3C and 3D). No other differences were found to be significant ($P > 0.05$).

DISCUSSION

In the current study, the daily minimum and maximum environmental temperature and RH were, on average, 22°C and 36°C, and 28% and 56%, respectively. We observed a change in the behavior of the birds, which started panting during the high

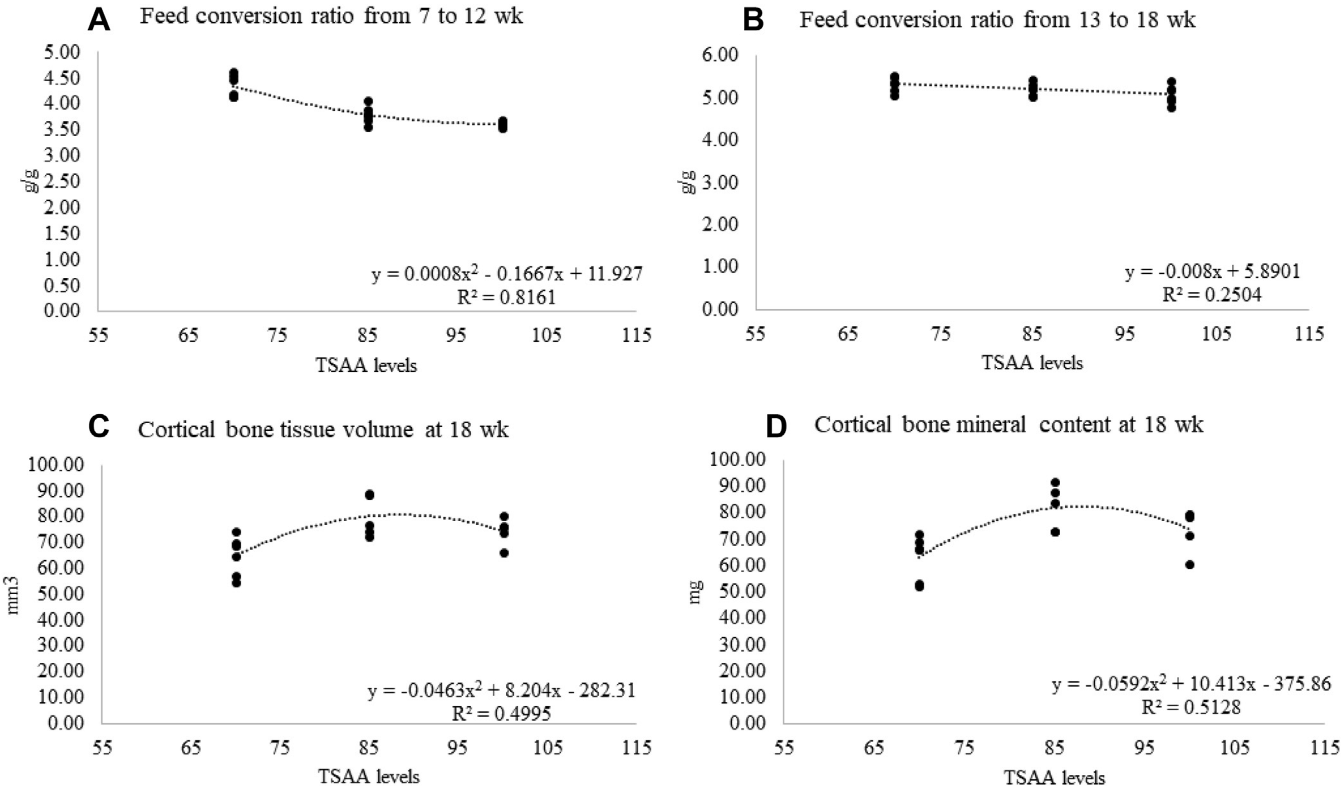

Figure 3. Feed conversion ratio from 7 to 12 (A) and 13 to 18 wk (B), cortical bone tissue volume (C), and cortical bone mineral content at 18 wk (D) according to TSAA levels. Abbreviation: TSAA, total sulfur amino acid.

temperature hours, and an increase, on average, of approximately 1°C in body temperature (T_b) when compared with the T_b during the non-HS hours (41.2°C at 9 AM and 42.1°C at 3 PM). Similarly, an increase in rectal temperature of 0.8°C was reported by Chen et al. (2013), when pullets were subjected to an environmental temperature of 35°C. Additionally, in general, the pullets showed a reduction of 11.5% in BW and 8.4% in FI compared with the expected results based on the HyLine W36 guideline (2016). Similar reduction in BW and FI in heat stressed birds was observed by Mashaly et al. (2004).

One of the most well-documented effects of HS in animal production is the decrease in performance. An essential mechanism to maintain T_b within a physiological safe range under HS is to reduce heat production related to consumption and metabolic utilization of feed; thus, the birds protect themselves through anorexia (Dale and Fuller, 1980; Temim et al., 2000; Mashaly et al., 2004; Franco-Jimenez et al., 2007). With the reduction in FI, fewer nutrients are available for maintenance and production. Dale and Fuller (1980) suggested that approximately 63% of growth depression in broilers due to HS is directly related to reduced FI. The other 37% can be associated to a direct effect of high temperature on reproductive physiology, health, energy metabolism, and protein and fat deposition (Renaudeau et al., 2012). Therefore, based on the behavioral signs such as increase in body temperature and reduction in performance, we affirmed that the birds in the present study were under HS.

Most importantly, the growth performance and bone development were also affected by the TSAA levels. As expected, increasing levels of TSAA in the diet led to higher TSAA intake compared with birds fed TSAA deficient diet (70% of TSAA), and this increase followed a linear fashion. The birds fed the diet deficient in TSAA showed reduced BWG and higher FCR than the ones fed higher levels of TSAA. These findings agree with Novak et al. (2004) and Gomes et al. (2011) that have shown quadratic and linear increases of BWG and a linear decrease in feed efficiency, with increased levels of TSAA in laying hens and broiler breeders.

The calculated TSAA levels for maximum BWG (95% of TSAA) found in our study are slightly lower than the line guideline requirement (considered as 100% of TSAA), suggesting that L-Met reduces Met requirement to achieve the maximum growth in pullets under HS. This finding is in accordance to another study conducted by our research group, in which we observed that, under high temperature, there is not an increase in the TSAA requirement for laying hens (Castro et al., 2019). The calculated TSAA level for maximum BWG found in our study from 1 to 6 wk (0.793% of TSAA) was slightly higher than the level suggested by D'Agostini et al. (2017), which was 0.778 of TSAA. Additionally, the calculated TSAA levels for minimum FCR were 99.08 and 104.18% of the levels recommended by the line guideline during 1 to 6 and 7 to 12 wk, respectively. According to Schutte et al. (1994), the TSAA levels for obtaining maximum efficiency of feed utilization are

Table 3. Means of bone weight, ash, collagenous protein (ColP), noncollagenous protein (NColP), tissue volume (TV), bone mineral content (BMC), and bone mineral density (BMD) from total bone, cortical, and trabecular bones according to TSAA levels at 12 wk of age.

	TSAA levels (%)			*P*-value		
Traits	70%	85%	100%	ANOVA	Regression	SE
Weight (g)	3.326	3.595	3.951	NS	NS	0.1188
Ash (g)	1.491	1.709[1]	1.833[1]	<0.0001	NS	0.0409
ColP (%)	40.167	33.235[1]	28.484[1]	0.0019	NS	1.5876
ColP (g)	0.569	0.544	0.521	NS	NS	0.0096
NColP (%)	0.492	0.412[1]	0.458	0.0498	NS	0.0140
NColP (mg)	7.011	7.167	8.572[1]	0.0275	NS	0.2872
Total bone TV (mm^3)	247.290	280.110[1]	291.650[1]	0.0054	NS	6.7643
Total BMC (mg)	65.728	74.968[1]	77.562[1]	0.0038	NS	1.7853
Total BMD (mg/mm^3)	0.266	0.268	0.266	NS	NS	0.0038
Cortical TV (mm^3)	62.767	69.313	72.046[1]	0.0446	NS	1.6853
Cortical BMC (mg)	50.751	55.391	57.308[1]	0.0380	NS	1.1692
Cortical BMD (mg/mm^3)	0.809	0.799	0.797	NS	NS	0.0076
Trabecular TV (mm^3)	9.368	11.418	12.583[1]	0.0351	NS	0.5627
Trabecular BMC (mg)	5.863	7.253	7.812[1]	0.0392	NS	0.3490
Trabecular BMD (mg/mm^3)	0.626	0.636	0.621	NS	NS	0.0038

Abbreviations: NS, not significant; TSAA, total sulfur amino acid.
N = 6.
[1] Means differ from 70% of TSAA by Dunnett test ($P \leq 0.05$).

higher than the levels for maximum production in laying hens.

The development of structural bone in laying hens happens during the pullet phase, and it is completed by the onset of sexual maturity. This growth process involves a complex relationship between mature chondrocytes, osteoblasts, and osteoclasts (Whitehead, 2004). These cells will produce the extracellular matrix, which is rich in collagen, proteoglycans, and growth factors, and initiate the mineralization and remodeling that take place in developing bone (Whitehead, 2004). Therefore, it is important to evaluate both organic and inorganic portions of the bone to assess its quality.

In the present study, at 12 wk of age, the ColP in the bones was higher when birds were fed 70% of TSAA compared with the other treatments. The ColP values were given as a percentage of the bone weight, and birds fed TSAA-deficient diet had bones which were 7.5 and 15.82% lighter than the ones from birds fed 85 and 100% of TSAA, respectively. Moreover, no statistical difference was observed between the treatments concerning the amount of ColP (g). Similarly, the NColP (%) was higher for birds fed 70% compared with 85% of TSAA; however, no differences were found between these 2 treatments regarding the total amount of NColP (mg). This suggests that there was no real increase in ColP and NColP in birds fed 70% of TSAA, and the results were mostly influenced by the bone size. Interestingly, a study *in vitro* using mice cells showed that a TSAA restriction led to a downregulation in gene expression of markers for collagen formation and bone differentiation (Ouattara et al., 2016). However, we

Table 4. Means estimates of bone weight, ash, collagenous protein (CP), noncollagenous protein (NCP), tissue volume (TV), bone mineral content (BMC), and bone mineral density (BMD) from total bone, cortical, and trabecular bones according to TSAA levels at 18 wk of age.

	TSAA levels (%)[1]			*P*-value				
Traits	70%	85%	100%	ANOVA	Regression[1]	Maximum response (%)	R^2	SE
Weight (g)	4.652	5.042[2]	5.078[2]	0.0485	NS	-	-	0.0836
Ash (g)	2.202	2.410[2]	2.438[2]	0.0083	NS	-	-	0.0386
ColP (%)	18.649	14.275	15.048	NS	NS	-	-	0.9533
ColP (g)	0.441	0.405	0.425	NS	NS	-	-	0.0185
NColP (%)	0.208	0.226	0.199	NS	NS	-	-	0.0118
NColP (mg)	5.103	6.753	5.676	NS	NS	-	-	0.3151
Total bone TV (mm^3)	281.160	311.230[2]	301.110	0.0468	NS	-	-	5.3716
Total BMC (mg)	98.330	114.110	96.660	NS	NS	-	-	6.3856
Total BMD (mg/mm^3)	0.351	0.364	0.321	NS	NS	-	-	0.0197
Cortical TV (mm^3)	64.855	80.154[2]	74.596	0.0111	0.0183[Q]	88.59	0.50	2.3553
Cortical BMC (mg)	63.155	81.794[2]	73.809	0.0093	0.0116[Q]	87.94	0.51	2.7986
Cortical BMD (mg/mm^3)	0.973	1.021	0.988	NS	NS	-	-	0.0135
Trabecular TV (mm^3)	5.894	9.20	11.881	NS	NS	-	-	1.1490
Trabecular BMC (mg)	4.929	7.808	9.951	NS	NS	-	-	1.0137
Trabecular BMD (mg/mm^3)	0.816	0.842	0.824	NS	NS	-	-	0.0090

Abbreviations: ColP, collagenous proteins; NColP, noncollagenous proteins; NS, not significant; TSAA, total sulfur amino acid.
N = 6.
[1] Quadratic (Q).
[2] Means differ from 70% of TSAA by Dunnett test ($P \leq 0.05$).

did not perform any additional *in vitro* analyses in the present study, but our findings suggest that the TSAA restriction did not alter the synthesis of ColP.

We hypothesized that, in pullets, the use of a diet deficient in TSAA (70% of TSAA) led to a delayed bone growth and mineralization. The lower ash, lower total bone TV, and total BMC in bones of pullets fed 70% of TSAA compared with 85 and 100% at 12 wk of age are evidence of this poor bone development. At this age, the ash contents were 12.8 and 18.7% higher in birds fed 85 and 100% of TSAA, respectively, compared with 70% of TSAA. Additionally, the total bone TV, which is indicative of bone size, was 11.7 and 15.2% higher when 85 and 100% of TSAA were used, respectively, compared with 70% of TSAA. Similarly, at 18 wk, we continued to observe poor mineralization and growth of tibias from pullets fed 70% of TSAA. The ash contents were 8.7 and 9.7% higher in birds fed 85 and 100% of TSAA, respectively, compared with 70% of TSAA, and the bone weights were 7.7 and 8.4% higher for 85 and 100%, respectively. In our study, the FI of birds fed 70% of TSAA was statistically similar to birds fed 85 and 100% of TSAA. Therefore, we cannot determine that the poor bone development observed in the deficient group was due to lower mineral intake.

Furthermore, when the cortical and trabecular bones were analyzed separately using MicroCT at 12 wk, the results showed that the use of 100% of TSAA led to higher cortical TV and BMC, and trabecular TV and BMC than 70% of TSAA, indicating that pullets fed 100% of TSAA had a better formation of structural bone compared with pullets fed deficient TSAA diet under HS. This result is reinforced by the NColP content (mg) in the bones of birds fed 100%, which was 18.22% higher than the content of bones from birds fed 70% of TSAA. Interestingly, at 18 wk, the total bone TV, cortical TV, and BMC were only higher for birds fed 85% of TSAA compared with 70% of TSAA. We also found a quadratic effect of TSAA levels on cortical TV and cortical BMC, and the level to reach maximum values for both traits was approximately 88% of the line TSAA recommendation. This result may be because of the onset of egg production and, consequently, increased medullary bone formation in birds fed 100% of TSAA at the expense of cortical and trabecular bones. To the best of our knowledge, there are no studies evaluating bone quality and TSAA levels in pullets.

Based on our findings, we conclude that the use of a TSAA-deficient diet (70% of TSAA) leads to poor growth performance and to a delay in bone development and mineralization, compared with the other TSAA levels. However, it appears that the use of 100% of TSAA is more beneficial, leading to better initial structural bone development. Furthermore, the level for achieving maximum BWG was 95% of TSAA, which is lower than the recommended TSAA level (100% of TSAA). Therefore, the TSAA level recommended by the line guideline or 95% of TSAA could be sufficient to assure good growth performance and bone quality in pullets under HS.

ACKNOWLEDGMENTS

The authors thank CJ Corporation for the financial support.

Conflict of Interest Statement: The authors did not provide any conflict of interest statement.

REFERENCES

AOAC International. 1990. Vitamin D3 in poultry supplements. In Official Methods of Analysis of AOAC International. 15th ed. AOAC International, Arlington, VA.

Attia, Y. A., A. E.-H. E. Abd El-Hamid, A. A. Abedalla, M. A. Berika, M. A. Al-Harthi, O. Kucuk, K. Sahin, and B. M. Abou-Shehema. 2016. Laying performance, digestibility and plasma hormones in laying hens exposed to chronic heat stress as affected by betaine, vitamin C, and/or vitamin E supplementation. SpringerPlus. Accessed Jan. 2019. http://springerplus.springeropen.com/articles/10.1186/s40064-016-3304-0.

Baines, M., M. B. Kredan, A. Davison, G. Higgins, C. West, W. D. Fraser, and L. R. Ranganath. 2007. The association between Cysteine, bone Turnover, and Low bone mass. Calcified Tissue Int. 81:450–454.

Barbosa, A. de A., G. H. K. de Moraes, R. de A. Torres, D. T. da C. Reis, C. de S. Rodrigues, and E. S. Müller. 2010. Avaliação da qualidade óssea mediante parâmetros morfométricos, bioquímicos e biomecânicos em frangos de corte. Revista Brasileira de Zootecnia 39:772–778.

Blewett, H. J. H. 2008. Exploring the mechanisms behind S-Adenosylmethionine (SAMe) in the treatment of Osteoarthritis. Crit. Rev. Food Sci. Nutr. 48:458–463.

Bradford, M. M. 1976. A Rapid and Sensitive method for the Quantitation of Microgram Quantities of protein utilizing the Principle of protein-Dye Binding. Anal. Biochem. 72:248–254.

Bunchasak, C. 2009. Role of dietary methionine in poultry production. J. Poult. Sci. 46:169–179.

Bunchasak, C., and T. Silapasorn. 2005. Effects of adding methionine in Low-protein diet on production performance, reproductive Organs and Chemical Liver Composition of laying hens under Tropical Conditions. Int. J. Poult. Sci. 4:301–308.

Castro, F. L. S., H. Y. Kim, Y. G. Hong, and W. K. Kim. 2019. The effect of total sulfur amino acid levels on growth performance, egg quality, and bone metabolism in laying hens subjected to high environmental temperature. Poult. Sci. 98:4982–4993.

Chen, X. Y., P. P. Wei, S. Y. Xu, Z. Y. Geng, and R. S. Jiang. 2013. Rectal temperature as an indicator for heat tolerance in chickens. Anim. Sci. J. 84:737–739.

D'Agostini, P., P. C. Gomes, H. H. de C. Mello, A. A. Calderano, L. M. Sá, H. S. Rostagno, and L. F. T. Albino. 2017. Exigência de metionina + cistina para frangas de reposição na fase inicial (1 a 6 semanas de idade). Ciência Anim. Brasileira 18:1–12.

Dale, N. M., and H. L. Fuller. 1980. Effect of diet Composition on feed intake and growth of chicks under heat stress.: II. Constant vs. Cycling temperatures. Poult. Sci. 59:1434–1441.

Del Vesco, A. P., E. Gasparino, D. de O. Grieser, V. Zancanela, M. A. M. Soares, and A. R. de Oliveira Neto. 2015. Effects of methionine supplementation on the expression of oxidative stress-related genes in acute heat stress-exposed broilers. Br. J. Nutr. 113:549–559.

Franco-Jimenez, D. J., S. E. Scheideler, R. J. Kittok, T. M. Brown-Brandl, L. R. Robeson, H. Taira, and M. M. Beck. 2007. Differential effects of heat stress in three Strains of laying hens. J. Appl. Poult. Res. 16:628–634.

Gomes, P. C., C. A. R. de Lima, A. A. Calderano, H. S. Rostagno, and L. F. T. Albino. 2011. Effect of dietary levels of methionine + cystine on performance of broiler breeders. Revista Brasileira de Zootecnia 40:1014–1018.

Hy-Line W36, C. L 2016. Management guide. Accessed Jan. 2017. https://www.hyline.com/UserDocs/Pages/36_COM_ENG.pdf.

Mashaly, M. M., G. L. Hendricks, M. A. Kalama, A. E. Gehad, A. O. Abbas, and P. H. Patterson. 2004. Effect of heat stress on production parameters and Immune responses of Commercial laying hens. Poult. Sci. 83:889–894.

Métayer, S., I. Seiliez, A. Collin, S. Duchêne, Y. Mercier, P.-A. Geraert, and S. Tesseraud. 2008. Mechanisms through which sulfur amino acids control protein metabolism and oxidative status. J. Nutr. Biochem. 19:207–215.

Narvaez-Solarte, W., H. S. Rostagno, P. R. Soares, L. F. Uribe-Velasquez, and M. A. Silva. 2006. Nutritional requirement of Calcium in white laying hens from 46 to 62 Wk of age. Int. J. Poult. Sci. 5:181–184.

Novak, C., H. Yakout, and S. Scheideler. 2004. The Combined effects of dietary Lysine and total sulfur amino acid level on egg production parameters and egg components in Dekalb Delta laying hens. Poult. Sci. 83:977–984.

Ouattara, A., D. Cooke, R. Gopalakrishnan, T. Huang, and G. P. Ables. 2016. Methionine restriction alters bone morphology and affects osteoblast differentiation. Bone Rep. 5:33–42.

Renaudeau, D., A. Collin, S. Yahav, V. de Basilio, J. L. Gourdine, and R. J. Collier. 2012. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animal 6:707–728.

SAS Institute. 2017. SAS University Edition, Cary, NC.

Schutte, J. B., J. De Jong, and H. L. Bertram. 1994. Requirement of the laying hen for sulfur amino acids. Poult. Sci. 73:274–280.

Stipanuk, M. H. 2004. Sulfur amino acid metabolism: Pathways for production and Removal of Homocysteine and Cysteine. Annu. Rev. Nutr. 24:539–577.

Temim, S., A. M. Chagneau, S. Guillaumin, J. Michel, R. Peresson, and S. Tesseraud. 2000. Does excess dietary protein improve growth performance and carcass characteristics in heat-exposed chickens? Poult. Sci. 79:312–317.

Wallis, I. R., and D. Balnave. 1984. The influence of environmental temperature, age and sex on the digestibility of amino acids in growing broiler chickens. Br. Poult. Sci. 25:401–407.

Whitehead, C. C. 2004. Overview of bone biology in the egg-laying hen. Poult. Sci. 83:193–199.

Whitehead, C. C., and R. H. Fleming. 2000. Osteoporosis in cage Layers. Poult. Sci. 79:1033–1041.

Zuprizal, M. L., A. M. Chagneau, and P. A. Geraert. 1993. Influence of Ambient temperature on True digestibility of protein and amino acids of Rapeseed and soybean meals in broilers. Poult. Sci. 72:289–295.