p-ISSN 2615-787X e-ISSN 2615-790X
Accredited by Directorate General of Strengthening for Research and Development No: 30/E/KPT/2018

Tropical Animal Science Journal, September 2022, 45(3):356-367
DOI: https://doi.org/10.5398/tasj.2022.45.3.356
Available online at http://journal.ipb.ac.id/index.php/tasj

Bone Measurements of Arginine Deficient Broilers Growing under Warm Temperatures

H. T. Dao[a,b], A. F. Moss[a], E. J. Bradbury[c], & R. A. Swick[a,*]
[a]School of Environmental and Rural Science, Faculty of Science, Agriculture, Business and Law, University of New England
Armidale, New South Wales, 2351, Australia
[b]Faculty of Animal Science, Vietnam National University of Agriculture
Trau Quy Town, Gia Lam District, Hanoi, 100000, Vietnam
[c]Ridley AgriProducts, Melbourne, Victoria, 3000, Australia
*Corresponding author: rswick@une.edu.au
(Received 17-10-2021; Revised 15-12-2021; Accepted 18-01-2022)

ABSTRACT

The effects of L-arginine (Arg) and L-citrulline (Cit) supplementations in an Arg-deficient reduced-protein diet on tibia morphology and mineral composition were investigated in broilers maintained at the thermo-neutral (NT) and cyclic warm temperature (WT). Seven hundred and twenty Ross 308 male broilers were brooded together for the first 7 days and randomly assigned to four dietary treatments with 12 replicates of 15 birds each from days 8 to 35. The dietary treatments were standard protein diet (SP) with 22.3% and 20.9% crude protein in grower and finisher, respectively, an Arg-deficient reduced-protein diet with 2.5% lower protein (RP), and RP added with Arg (RP-Arg) or Cit (RP-Cit) at 0.28%. Dietary treatments were fed from day 8 with average bird's body weights of 177±3.25 g. Cyclic warm temperature (33 °C ± 1 °C for 6 h per day) was applied in one of the climate-controlled rooms during the finisher phase (21 to 35 day), resulting in a 2 × 4 factorial arrangement of treatments with the factors were the dietary treatment and temperature. Birds fed the RP diet had lower tibia breaking strength (day 28), ash (day 35), and diameter (days 21 and 28) compared to those offered the SP diet (p<0.05). Birds fed the RP diet had lower serum K and tibia B and higher tibia Mn level on day 21; higher serum Ca, P, and Mg, and lower tibia B level on day 28 compared to the SP-fed birds (p<0.05). Supplementation with Arg or Cit compensated for the adverse effects of the RP diet on these traits (p<0.05). Interactions showed tibia diameter decreased in birds fed the SP diet compared to the RP and RP-Cit diets only when raised under cyclic WT on day 35 (p<0.05). Thus, supplementation of Arg or Cit to the RP diet was necessary to support bone morphology and mineralisation under normal and warm temperatures.

Keywords: arginine; citrulline; minerals; heat stress; reduced protein

INTRODUCTION

Heat stress is a major concern in poultry production. The devastating effects of heat stress on bird growth rate and welfare have been documented by Belhadj *et al.* (2016) and Castro *et al.* (2019a). Furthermore, heat stress has been reported to reduce bone weight, ash content, and breaking strength resulting in considerable skeletal problems and lameness in broiler chickens (Sgavioli *et al.*, 2016; Mosleh *et al.*, 2018). Metabolism of protein generates more heat production than that of fat or carbohydrate (Wu *et al.*, 2019). Thus, reducing dietary crude protein (CP) level with supplementation of crystalline amino acids (AA) during heat stress may improve growth performance in birds (Zaman *et al.*, 2008). Effects of feeding reduced-protein diets on growth performance, carcass yield, and nitrogen digestibility have been extensively investigated (Belloir *et al.*, 2017; Hilliar *et al.*, 2019; Chrystal *et al.*, 2020; Dao *et al.*, 2021a). However, there were not many reports investigating the effects of feeding reduced-protein diets on bone morphology, strength, and mineralisation in birds during heat stress. Available data have shown that reducing dietary protein levels could increase serum Ca, Na, and Zn levels but decrease tibia Mn and Cu levels in broilers raised under standard conditions (Cowieson *et al.*, 2020). Bone quality is one of the most important indicators to evaluate the broiler's health and welfare (Brickett *et al.*, 2007). Furthermore, reduced bone strength might cause fractures during catching and transportation, increasing problems during processing and compromising economic returns (Talaty *et al.*, 2009). Hence, determining the possible effects of feeding reduced-protein diets on bone quality, such as bone morphology, strength, and mineralization, is necessary to expand the adoption of reduced-protein diets at the industrial level.

Arginine (Arg) is an essential amino acid for chickens as they do not have much, if any *de-novo* Arg synthesis (Fouad *et al.*, 2013). Therefore, chickens are strongly dependent on exogenous Arg sources provided from the diets. Aside from various important roles in protein synthesis and immunity, dietary Arg can also affect bone development by engaging in collagen and connective tissue formation (Jahanian, 2009). A recent study by Castro *et al.* (2019b) indicated that birds fed Arg-deficient diets exhibited lower bone mineral density compared to those fed the diets with sufficient levels of Arg. Arginine supplementation is recommended during the warm period when demand for this AA increases (Attia *et al.,* 2011; Esser *et al.*, 2017). Previous studies have shown that citrulline (Cit), a metabolite of Arg, could spare Arg requirements in broilers that may occur in the kidneys and other extrahepatic tissues through Arg-succinate formation (Su & Austic, 1999). Furthermore, Cit supplementation could decrease the mortality rate in pre-weaning piglets raised under warm temperatures (Kvidera *et al.,* 2016; Liu *et al.*, 2019). Thus, Cit supplementation may also decrease the mortality rate and improve the health of broilers under heat stress conditions. Growth performance, carcass traits, organ weights, ileal nutrient digestibility, serum uric acid, gut permeability, and femur morphology were reported by Dao *et al.* (2021b). This paper presents the effects of Arg and Cit supplementation in an Arg-deficient reduced-protein diet on tibia morphology, strength, and mineralisation of broilers housed under the same conditions. This study represents additional work of previously conducted research by Dao *et al.* (2021b), which aimed at determining the effects of Arg and Cit supplementation in an Arg-deficient reduced-protein diet in broilers maintained at the thermoneutral (NT) or cyclic warm temperature (WT).

MATERIALS AND METHODS

Protocol and Ethic

The study was approved by the Animal Ethics Committee of the University of New England (approval number: AEC19-036) and satisfied the requirements of the Australian government regarding the care and use of animals in research (NHMRC, 2013). Detailed information on the management, experimental design, dietary treatments, and feed analysis is described in Dao *et al.* (2021b).

Experimental Animals and Diets

Seven hundred and twenty day-old Ross 308 vent sexed males were brooded together and fed a common starter diet containing 3000 kcal/kg MEn, 24.6% CP, and sufficient mineral levels for the first 7 days in a temperature-controlled room. On day 8, birds were weighed (177±3.25 g) and evenly distributed to 48 equal-sized floor pens (120 × 80 cm) in two temperature-controlled rooms with 15 birds per pen. Birds had free access to the feed and water throughout the study. The temperature followed Ross 308 recommendations from days 0 to 21 (Aviagen, 2014a). From days 21 to 35, the temperature in one of the climate-controlled rooms was increased to 33 ± 1 ^{o}C for 6 h per day (WT), while the temperature in the NT room was maintained at 24 ^{o}C. The dietary treatments were: a standard protein diet (SP), an Arg-deficient reduced-protein diet (RP), RP added with Arg (RP-Arg), and RP added with Cit (RP-Cit). Arginine level in the RP-Arg diet and Cit level in the RP-Cit diet were equivalent to Arg level in the SP diet. The CP levels in the RP diets were approximately 2.5% lower than that in the SP diets. Arginine and Cit were added to the RP diets at 0.29% and 0.28% in the grower (days 7 to 21) and finisher (days 21 to 35) phases, respectively. All the diets met the energy requirement of Ross 308 broilers (Aviagen, 2014b). AMINOChick® 2.0 software (Evonik Animal Nutrition) was used to calculate the AA requirement of the birds. Detailed information on diet composition and nutrient contents are shown in Tables 1 and 2. These Tables were also published in Dao *et al.* (2021b). Supplemental Arg and Cit levels were determined following the procedures previously described by Dao *et al.* (2021c). The mineral composition of the dietary treatments is presented in Table 3. Generally, the mixed diets satisfied formulation objectives regarding mineral levels. Levels of Ca, P, K, Mg, S, Al, B, and Zn in the SP diet were generally higher than those of RP diets. Meanwhile, levels of Fe in all RP diets were higher than in the SP diet. Levels of other minerals were similar between the SP and RP diets.

Data Collection and Measurement

On days 21, 28, and 35, two birds per pen were randomly selected, weighed, electrically stunned, and decapitated to collect blood and tibias (from the right leg). Vacutainers (Becton, Dickinson U.K. Limited, Plymouth, UK) were used to collect the blood samples. Then the samples were centrifuged at 3000 × g at 4 °C for 10 minutes to collect the serum. The concentrations of Na, K, Ca, Mg, and P were measured in duplicate using the commercial kits in a Thermo Scientific™ Indiko™ Plus clinical chemistry analyser (Thermo Fisher Scientific Inc., Waltham, MA, US) following the manufacturer's instruction. The kits used were Sodium (Na) Enzymatic Colorimetric Test (catalogue number. NA 3851, Randox Laboratories Ltd, County Antrim, UK), Potassium (K) U.V. Test (catalogue number. PT 3852, Randox Laboratories Ltd, County Antrim, UK), Calcium (Reference number: 981772, Thermo Fisher Scientific Inc., Waltham, MA, US), Magnesium (Reference number: 981905, Thermo Fisher Scientific Inc., Waltham, MA, US), Phosphorus (Reference number: 981890, Thermo Fisher Scientific Inc., Waltham, MA, US). In addition, the blood serum Zn level was determined using a Zinc (Zn) kit (catalogue number. ZN 2341, Randox Laboratories Ltd, County Antrim, UK) following the manufacturer's instruction, and the results were read on a SpectraMax M2e plate reader (Molecular Devices, California, USA).

Tibias were collected and subjected to breaking strength, ash, and morphological (weight, length, diam-

Table 1. Composition and calculated nutrient values of standard and reduced-protein diets fed to broiler chickens (as-fed basis)

Ingredients	Starter	Grower		Finisher	
		SP[1]	RP[2]	SP	RP
Wheat (%)	52.31	52.10	67.11	56.72	69.56
Sorghum (%)	10.00	15.00	15.00	15.00	15.00
Meat and bone meal (%)	2.64	2.02	2.44	1.74	2.03
Canola meal (%)	5.00	8.00	1.30	8.00	5.18
Soybean meal (%)	26.07	18.40	10.47	14.33	4.83
Canola oil (%)	1.69	2.03	0.55	2.43	1.03
Limestone (%)	0.79	0.77	0.79	0.77	0.78
Salt (%)	0.26	0.19	0.19	0.17	0.17
Sodium bicarbonate (%)	0.24	0.20	0.20	0.15	0.15
Phytase (%)[3]	0.01	0.01	0.01	0.01	0.01
TiO_2 (%)	-	0.50	0.50	-	-
Vitamin premix (%)[4]	0.09	0.09	0.09	0.09	0.09
Mineral premix (%)[5]	0.12	0.12	0.12	0.12	0.12
Choline Cl 60% (%)	0.06	0.07	0.10	0.06	0.09
L-lysine (%)	0.35	0.30	0.59	0.25	0.54
D,L-methionine (%)	0.26	0.16	0.22	0.12	0.15
L-threonine (%)	0.09	0.05	0.19	0.04	0.16
L-isoleucine (%)	-	-	0.14	-	0.12
Albac 150 (ZnBac, %)	0.03	-	-	-	-
Calculated nutrient					
Dry matter (%)	91.52	91.58	91.39	91.53	91.42
Energy (kcal MEn/kg)	3,000	3,075	3,075	3,150	3,150
Crude protein (%)	24.61	22.29	19.50	20.86	18.36
Crude fat (%)	4.48	5.21	3.00	5.60	3.92
Crude fiber (%)	2.88	2.91	2.47	2.85	2.57
Ash (%)	4.56	4.41	3.82	3.52	2.96
Dig arginine (%)[6]	1.35	1.17	0.88	1.06	0.78
Dig lysine (%)	1.32	1.13	1.13	1.01	1.01
Dig methionine (%)	0.59	0.47	0.49	0.42	0.42
Dig cysteine	0.39	0.38	0.34	0.37	0.35
Dig TSAA (%)[7]	0.98	0.85	0.83	0.79	0.77
Dig tryptophan (%)	0.27	0.24	0.20	0.23	0.19
Dig histidine	0.55	0.50	0.42	0.47	0.40
Dig phenylalanine (%)	1.05	0.94	0.78	0.88	0.72
Dig leucine (%)	1.64	1.50	1.27	1.41	1.18
Dig isoleucine (%)	0.89	0.79	0.78	0.74	0.71
Dig threonine (%)	0.83	0.72	0.72	0.66	0.66
Dig valine (%)	1.13	1.04	0.92	1.00	0.88
Dig glycine (%)	0.99	0.88	0.77	0.83	0.72
Dig serine (%)	1.08	0.97	0.85	0.92	0.79
Calcium (%)	0.90	0.82	0.82	0.78	0.78
Available Phosphorus (%)	0.45	0.41	0.41	0.39	0.39
Sodium (%)	0.22	0.18	0.18	0.16	0.16
Chloride (%)	0.29	0.24	0.30	0.22	0.28
Choline (mg/kg)	1,700	1,600	1,600	1,500	1,500
Linoleic acid (%)	1.54	1.79	1.26	1.92	1.54
DEB (mEq/kg)[8]	272	238	188	214	167

Note: [1]Diet contained standard crude protein levels at 22.3% and 20.9% for grower and finisher phases, respectively. [2]Diet contained reduced crude protein levels at 19.5% and 18.4% for grower and finisher phases, respectively. L-arginine and L-citrulline were added to the RP diets at 0.29% and 0.28% for grower and finisher phases, respectively. [3]Phytase= Natuphos E 5000 G ((BASF Corporation, Florham Park, NJ, US) contains 500FTU/kg. [4]Vitamin premix per kg diet (UNE VM, Rabar Pty Ltd): vitamin A, 12 MIU; vitamin D, 5 MIU; vitamin E, 75 mg; vitamin K, 3 mg; nicotinic acid, 55 mg; pantothenic acid, 13 mg; folic acid, 2 mg; riboflavin, 8 mg; cyanocobalamin, 0.016 mg; biotin, 0.25 mg; pyridoxine, 5 mg; thiamine, 3 mg; antioxidant, 50 mg. [5]Mineral premix per kg diet (UNE TM, Rabar Pty Ltd): Cu, 16 mg as copper sulphate; Mn, 60 mg as manganese sulphate; Mn, 60 mg as manganous oxide; I, 0.125 mg as potassium iodide; Se, 0.3 mg; Fe, 40 mg, as iron sulphate; Zn, 50 mg as zinc oxide; Zn, 50 mg as zinc sulphate. [6]Digestible amino acid coefficients for raw ingredients were determined by Near-Infra Red spectroscopy (Foss NIR 6500, Denmark) standardized with Evonik AMINONIR® Advanced calibration. [7]Total sulphur amino acids (methionine + cysteine). [8]DEB (mEq/kg) calculated as 10.000 × (Na^+ + K^+ - Cl^-).

Table 2. Analysed nutrient values of experimental diets fed to broiler chickens (as-fed basis)

Nutrient composition	Grower								Finisher							
	SP[1]		RP[2]		RP-Arg[3]		RP-Cit[4]		SP		RP		RP-Arg		RP-Cit	
Dry matter (%)	86.52		86.91		86.70		86.66		86.41		86.91		86.63		86.08	
Energy (kcal GE/kg)	3,945		3.825		3.855		3.840		3.975		3.894		3.914		3.907	
Crude protein (%)	22.20	(22.29)[5]	19.52	(19.50)	19.96	(20.0)	20.06	(20.0)	21.06	(20.86)	18.42	(18.36)	18.82	(18.86)	18.84	(18.86)
Crude fat (%)	3.29		1.64		1.64		1.64		4.20		2.48		1.64		1.64	
Crude fiber (%)	2.51		2.03		2.03		2.03		2.45		2.09		2.09		2.09	
Ash (%)	4.29		4.31		4.21		4.56		3.39		3.36		3.33		3.89	
Citrulline (%)	-		-		-		0.25		-		-		-		0.23	
Arginine (%)[6]	1.21	(1.30)	0.92	(1.01)	1.19	(1.30)	0.92	(1.01)	1.14	(1.19)	0.87	(0.90)	1.13	(1.18)	0.87	(0.90)
Lysine (%)	1.21	(1.27)	1.12	(1.24)	1.12	(1.24)	1.12	(1.24)	1.12	(1.14)	1.02	(1.11)	1.02	(1.11)	1.02	(1.11)
Methionine (%)	0.45	(0.51)	0.45	(0.53)	0.45	(0.53)	0.45	(0.53)	0.43	(0.46)	0.39	(0.45)	0.39	(0.45)	0.39	(0.45)
Cysteine (%)	0.40	(0.45)	0.34	(0.39)	0.34	(0.39)	0.34	(0.39)	0.42	(0.44)	0.36	(0.40)	0.36	(0.40)	0.36	(0.40)
TSAA (%)[7]	0.85	(0.96)	0.79	(0.92)	0.79	(0.92)	0.79	(0.92)	0.85	(0.90)	0.75	(0.85)	0.75	(0.85)	0.75	(0.85)
Tryptophan (%)	0.26	(0.28)	0.21	(0.23)	0.21	(0.23)	0.21	(0.23)	0.25	(0.27)	0.20	(0.22)	0.20	(0.22)	0.20	(0.22)
Histidine (%)	0.51	(0.57)	0.41	(0.47)	0.41	(0.47)	0.41	(0.47)	0.49	(0.53)	0.39	(0.45)	0.39	(0.45)	0.39	(0.45)
Phenylalanine (%)	1.01	(1.06)	0.85	(0.87)	0.85	(0.87)	0.85	(0.87)	0.96	(0.99)	0.80	(0.81)	0.80	(0.81)	0.80	(0.81)
Leucine (%)	1.60	(1.71)	1.34	(1.43)	1.34	(1.43)	1.34	(1.43)	1.52	(1.61)	1.26	(1.34)	1.26	(1.34)	1.26	(1.34)
Isoleucine (%)	0.90	(0.90)	0.82	(0.85)	0.82	(0.85)	0.82	(0.85)	0.82	(0.83)	0.77	(0.78)	0.77	(0.78)	0.77	(0.78)
Threonine (%)	0.79	(0.86)	0.70	(0.82)	0.70	(0.82)	0.70	(0.82)	0.73	(0.79)	0.66	(0.76)	0.66	(0.76)	0.66	(0.76)
Valine (%)	0.99	(1.20)	0.81	(1.03)	0.81	(1.03)	0.81	(1.03)	0.94	(1.14)	0.77	(1.00)	0.77	(1.00)	0.77	(1.00)
Glycine (%)	0.95	(1.08)	0.80	(0.94)	0.80	(0.94)	0.80	(0.94)	0.96	(1.01)	0.80	(0.88)	0.80	(0.88)	0.80	(0.88)
Taurine (%)	0.13		0.15		0.15		0.15		0.14		0.14		0.14		0.14	
Serine (%)	0.84	(1.12)	0.72	(0.95)	0.72	(0.95)	0.72	(0.95)	0.83	(1.05)	0.68	(0.89)	0.68	(0.89)	0.68	(0.89)
Glutamic acid (%)	4.81	(4.88)	4.57	(4.47)	4.57	(4.47)	4.57	(4.47)	4.76	(4.73)	4.40	(4.33)	4.40	(4.33)	4.40	(4.33)
Proline (%)	1.57	(1.50)	1.48	(1.38)	1.48	(1.38)	1.48	(1.38)	1.57	(1.45)	1.47	(1.35)	1.47	(1.35)	1.47	(1.35)
Alanine (%)	0.94	(1.04)	0.79	(0.87)	0.79	(0.87)	0.79	(0.87)	0.92	(0.97)	0.75	(0.82)	0.75	(0.82)	0.75	(0.82)
Tyrosine (%)	0.64	(0.65)	0.53	(0.59)	0.53	(0.59)	0.53	(0.59)	0.61	(0.60)	0.50	(0.50)	0.50	(0.50)	0.50	(0.50)

Note: [1]Diet contained standard crude protein levels at 22.3% and 20.9% for grower and finisher phases, respectively. [2]Diet contained reduced crude protein levels at 19.5% and 18.4% for grower and finisher phases, respectively. [3]Reduced-protein diet supplemented with 0.29% and 0.28% L-arginine for grower and finisher phases, respectively. [4]Reduced-protein diet supplemented with 0.29% and 0.28% L-citrulline for grower and finisher phases, respectively. [5]Values in brackets were calculated nutrients.[6]Values of all amino acids presented were total amino acids.[7]Total sulphur amino acids (methionine + cysteine).

Table 3. Analysed mineral composition of dietary treatments fed to broiler chickens

Dietary treatments		Ca (mg/g)	P (mg/g)	Na (mg/g)	Mg (mg/g)	K (mg/g)	S (mg/g)	Al (mg/kg)	B (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Mo (mg/kg)
Grower (days 7 to 21)	SP[1]	8.28	5.68	1.69	2.11	8.2	2.81	210	9.34	138	27.91	163	175	7.35
	RP[2]	8.21	5.32	1.71	1.78	6.44	2.57	186	5.52	177	27.16	152	170	7.65
	RP-Arg[3]	8.19	5.34	1.71	1.80	6.37	2.54	202	5.51	163	27.64	162	176	7.36
	RP-Cit[4]	8.16	5.30	1.67	1.76	6.21	2.50	195	5.25	164	28.19	159	172	7.35
Finisher (days 21 to 35)	SP	8.16	5.57	1.47	2.14	7.68	2.83	110	8.41	147	27.91	171	188	5.99
	RP	7.75	5.37	1.40	1.82	5.40	2.44	93	3.65	164	26.57	154	167	5.94
	RP-Arg	7.77	5.36	1.39	1.83	5.40	2.44	116	3.53	157	26.05	157	186	6.12
	RP-Cit	7.78	5.34	1.39	1.79	5.26	2.4	101	3.55	155	25.04	154	169	6.32

Note: [1]Diet contained standard crude protein levels at 22.3% and 20.9% for grower and finisher phase, respectively. [2]Diet contained reduced crude protein levels at 19.5% and 18.4% for grower and finisher phase, respectively. [3]Reduced-protein diet supplemented with 0.29% and 0.28% L-arginine in grower and finisher phases, respectively.[4]Reduced-protein diet supplemented with 0.29% and 0.28% L-citrulline in grower and finisher phases, respectively.

eter) measurements following the procedures described by Dao *et al.* (2021a). The bone Seedor index (mg/mm) was calculated by dividing the weight of oven-dry bone (mg) by the bone length (mm) as described by Seedor *et al.* (1991). The feed and tibia mineral composition were determined in an inductively coupled plasma-optical emission spectrometry instrument (Agilent, Victoria, Australia) using procedures previously described by Zanu *et al.* (2020).

Statistical Analysis

Statistical differences between the treatments were tested using R Commander (version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria) using either one-way ANOVA or the non-parametric ANOVA (Kruskal–Wallis test). In addition, the interaction between temperature and dietary treatments during the cyclic WT period (days 21 to 35) was tested using a two-way ANOVA. Differences among treatments were compared by Tukey's post-hoc tests where appropriate. Data were tested for normality and variance homogeneity before analysis. A significant difference was considered at p-value ≤ 0.05 and a tendency was considered at 0.05 < p-value < 0.10.

RESULTS

Serum Mineral Composition

Serum mineral composition in the treatments on days 21, 28, and 35 are shown in Tables 5, 7, 9, and 12. A marginal temperature × diet interaction was observed for serum Ca level on day 28 (p= 0.092, Table 7), indicating that serum Ca level tended to increase in birds fed the RP-Arg diet compared to the SP diet only when raised under cyclic WT. The cyclic WT as the main effect increased Ca level and decreased serum Na and K levels compared to the NT on day 28 (p<0.05, Tables 7 and 9). Similarly, lower serum Na level was observed in birds housed under cyclic WT compared to those housed under NT on day 35 (p<0.001, Table 12).

Supplementation of Arg to the RP diet tended to decrease serum Ca level compared to the RP on day 21 (p=0.062, Table 5). Birds fed the RP diet had higher serum Ca, P, and Mg level compared to those offered

Table 4. Tibia weight, ash, length, diameter, and breaking strength of broiler chickens on day 21

Dietary treatments	Variables						
	Absolute weight[5] (g)	Relative weight[6] (%)	Ash[7] (%)	Length[8] (mm)	Seedor index[9] (mg/mm)	Diameter (mm)	Breaking strength (N)
SP[1]	5.01	0.50[a]	47.54	66.15	32.23	6.18	285
RP[2]	4.73	0.53[b]	47.46	65.82	30.91	5.9	266
RP-Arg[3]	4.84	0.51[ab]	47.44	65.53	32.32	6.01	264
RP-Cit[4]	4.81	0.51[ab]	46.82	65.87	31.85	5.89	278
Pooled SEM	0.05	0.003	0.17	0.22	0.29	0.04	8.12
p-value	0.299	0.033	0.395	0.805	0.282	0.051	0.714

Note: [a,b] Differing superscripts within a column indicate significant differences between means. [1]Diet contained standard crude protein level at 22.3% for grower phase (days 7 to 21). [2]Diet contained reduced crude protein level at 19.5% for grower phase (days 7 to 21). [3]Reduced-protein diet supplemented with 0.29% L-arginine for grower phase (days 7 to 21). [4]Reduced-protein diet supplemented with 0.29% L-citrulline for grower phase (days 7 to 21). [5]Tibia weight was measured on the fresh tibia. [6]Relative tibia weight as a percentage of body weight (%). [7]Tibia ash was expressed as a percentage of the oven-dry tibia (%). [8]Tibia length, diameter, and breaking strength were measured on air-dry tibias. [9]Seedor index: weight of oven-dry tibia/tibia length (mg/mm).

Table 5. Serum mineral composition of broiler chickens on day 21 (mg/dL)

Dietary treatments	Variables					
	Ca	P	Na	Mg	K	Zn
SP[1]	8.77	6.19	392	2.21	33.58	0.176
RP[2]	9.24	6.44	388	2.22	30.29	0.172
RP-Arg[3]	8.30	6.34	391	2.20	32.06	0.179
RP-Cit[4]	8.63	6.40	391	2.31	33.14	0.184
Pooled SEM	0.13	0.08	1.53	0.03	0.50	0.004
p-value	0.062	0.689	0.891	0.416	0.080	0.783

Note: [a,b]Differing superscripts within a column indicate significant differences between means. [1]Diet contained standard crude protein level at 22.3% for grower phase (days 7 to 21). [2]Diet contained reduced crude protein level at 19.5% for grower phase (days 7 to 21). [3]Reduced-protein diet supplemented with 0.29% L-arginine for grower phase (days 7 to 21). [4]Reduced-protein diet supplemented with 0.29% L-citrulline for grower phase (days 7 to 21).

Table 6. Tibia mineral composition of broiler chickens on day 21

Dietary treatments	Variables											
	Ca (mg/g)	P (mg/g)	Na (mg/g)	Mg (mg/g)	K (mg/g)	S (mg/g)	Al (mg/kg)	B (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Mo (mg/kg)
SP[1]	386	178	13.99	8.10	8.49	4.27	17.01	6.10[b]	329	514	4.88[a]	32.66
RP[2]	385	178	13.9	8.38	8.12	4.21	15.61	4.40[a]	301	519	7.46[b]	31.35
RP-Arg[3]	385	177	13.79	8.10	8.17	3.96	16.52	4.22[a]	309	513	8.02[b]	32.21
RP-Cit[4]	385	177	13.72	8.20	8.13	4.02	15.83	4.06[a]	301	502	7.47[b]	32.17
Pooled SEM	0.62	0.32	0.07	0.05	0.06	0.05	0.33	0.13	4.41	3.10	0.26	0.44
p-value	0.738	0.672	0.58	0.10	0.106	0.137	0.431	< 0.001	0.084	0.297	< 0.001	0.872

Note: [a,b]Differing superscripts within a column indicate significant differences between means. Cooper (Cu) was not detected in tibia on day 21. [1]Diet contained standard crude protein level at 22.3% for grower phase (days 7 to 21). [2]Diet contained reduced crude protein level at 19.5% for grower phase (days 7 to 21). [3]Reduced-protein diet supplemented with 0.29% L-arginine for grower phase (days 7 to 21). [4]Reduced-protein diet supplemented with 0.29% L-citrulline for grower phase (days 7 to 21).

the SP diet regardless of the room temperature on day 28 (p<0.05, Tables 7 and 9). Supplementation of either Arg or Cit to the RP diet decreased serum Ca and P to the levels of the birds offered the SP diet on day 28. Arginine supplementation to the RP diet increased serum Zn level compared to the SP treatment (p<0.05), and decreased serum Mg to the level that was not different to the serum Mg level of birds fed the SP diet on day 28 (Table 9). On day 35, birds fed the RP diet had higher serum Ca levels compared to those fed the SP diet (p<0.001, Table 12). Supplementation of Cit to the RP diet decreased serum Ca level compared to the RP (p<0.001), increased serum Mg level compared to the SP (p<0.05), and tended to increase serum P level compared to the SP on day 35 (p=0.063, Table 12).

Table 7. Variables with significant/marginal significant temperature × diet interactions on broiler chickens

Treatments		Day 28 tibia ash[7] (%)	Day 28 serum Ca (mg/dL)	Day 28 tibia Mn (mg/kg)	Day 35 tibia diameter (mm)
NT[1]	SP[3]	44.78	8.12	10.07	9.25[c]
	RP[4]	43.21	9.66	8.33	8.46[ab]
	RP-Arg[5]	45.27	8.18	10.47	8.82[bc]
	RP-Cit[6]	44.36	9.29	10.24	8.44[ab]
WT[2]	SP	45.14	8.79	4.78	8.44[ab]
	RP	44.42	10.37	7.28	8.06[a]
	RP-Arg	43.96	9.94	6.62	8.41[ab]
	RP-Cit	44.72	9.10	7.42	8.50[ab]
Mean of main effects					
Temperature	NT	44.4	8.81[a]	9.78[a]	8.74[a]
	WT	44.56	9.55[b]	6.53[b]	8.35[b]
Diet	SP	44.96	8.46[a]	7.43	8.84[b]
	RP	43.81	10.01[b]	7.81	8.26[a]
	RP-Arg	44.61	9.06[ab]	8.54	8.61[ab]
	RP-Cit	44.54	9.19[ab]	8.83	8.47[ab]
Pooled SEM		0.18	0.16	0.39	0.07
p-value	Temperature	0.672	0.023	< 0.001	0.003
	Diet	0.152	0.006	0.586	0.014
	Temperature × diet	0.083	0.092	0.092	0.045

Note: [a,b]Differing superscripts within a column indicate significant differences between means. [1]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 24 °C from days 21 to 35. [2]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 33±1 °C for 6 h per day from days 21 to 35. [3]Diet contained standard crude protein levels at 22.3% and 20.9% for grower and finisher phases, respectively. [4]Diet contained reduced crude protein levels at 19.5% and 18.4% for grower and finisher phases, respectively. [5]Reduced-protein diet supplemented with 0.29% and 0.28% L-arginine in grower and finisher phases, respectively. [6]Reduced-protein diet supplemented with 0.29% and 0.28% L-citrulline in grower and finisher phases, respectively. [7]Relative tibia ash as percentage of air-dry tibia (%).

Table 8. Tibia weight, length, diameter, and breaking strength of broiler chickens on day 28

Treatments		Variables					
		Absolute weight[7](g)	Relative weight[8] (%)	Length[9] (mm)	Seedor index[10] (mg/mm)	Diameter (mm)	Breaking strength (N)
Temperature	NT[1]	7.44	0.52	77.78	44.41	7.26	297
	WT[2]	7.14	0.51	77.2	43.50	7.15	280
Diet	SP[3]	7.53	0.520[ab]	77.36	45.10	7.45	318[b]
	RP[4]	7.24	0.528[b]	77.49	44.32	7.05	265[a]
	RP-Arg[5]	7.22	0.495[a]	77.55	43.56	7.22	287[ab]
	RP-Cit[6]	7.17	0.511[ab]	77.56	42.84	7.09	284[ab]
Pooled SEM		0.09	0.00	0.23	0.38	0.06	6.370
p-value	Temperature	0.093	0.229	0.211	0.232	0.324	0.170
	Diet	0.47	0.007	0.990	0.169	0.054	0.027
	Temperature × diet	0.68	0.572	0.379	0.742	0.568	0.106

Note: [a,b]Differing superscripts within a column indicate significant differences between means. [1]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 24 °C from days 21 to 35. [2]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 33±1 °C for 6 h per day from days 21 to 35. [3]Diet contained standard crude protein levels at 22.3% and 20.9% for grower and finisher phases, respectively. [4]Diet contained reduced crude protein levels at 19.5% and 18.4% for grower and finisher phases, respectively. [5]Reduced-protein diet supplemented with 0.29% and 0.28% L-arginine in grower and finisher phases, respectively. [6]Reduced-protein diet supplemented with 0.29% and 0.28% L-citrulline in grower and finisher phases, respectively. [7]Tibia weight was measured on the fresh tibia. [8]Relative tibia weight as a percentage of body weight. [9]Tibia length, diameter, and breaking strength were measured on air-dry tibias. [10]Seedor index= weight of oven-dry tibia/tibia length (mg/mm).

Tibia Mineral Composition

Tibia mineral composition on days 21, 28, and 35 are presented in Tables 6, 7, 10, and 13. A marginal temperature × diet interaction was observed for tibia Mn level on day 28 (p=0.092, Table 7), where tibia Mn level tended to increase in birds fed the RP-Cit diet compared to the SP diet only when raised under cyclic WT. The cyclic WT as the main effect reduced levels of Ca, P, Al, B, Cu, Mo, and Mn in the tibia compared to the control on day 28 (p<0.01; Tables 7 and 10). Lower tibia Mo level but higher tibia Ca, P, Al, B, and Zn levels were observed in cyclic WT birds compared to those housed under NT on day 35 (p<0.05, Table 13).

Birds fed the RP diet had lower B levels (p<0.001), higher Mn level (p<0.001), and tended to have lower Fe level (p=0.084) in the tibias compared to those offered the SP diet on day 21 (Table 6). Also, birds fed the RP diet exhibited lower tibia B levels compared to birds fed the SP diet on day 28, as shown by the main effect of dietary treatment in Table 10 (p<0.001). Supplementation of either Arg or Cit to the RP diet decreased the tibia Zn level compared to the RP diet on day 28 (p=0.001, Table 10). On day 35, levels of all examined minerals in the tibias were not different between the RP and SP diets (p>0.05, Table 13). Both Arg and Cit supplementation to the RP diet increased Mn levels in the respective groups compared to the SP on day 35 (p<0.01, Table 13). Supplementation of Arg to the RP diet decreased tibia Zn level compared to the RP and decreased tibia K level compared to the RP-Cit on day 35 (p=0.01). Supplementation of Cit to the RP diet increased tibia Na level compared to the RP on day 35 (p<0.05, Table 13).

Tibia Morphology and Strength

The effects of dietary treatments and cyclic WT on tibia weight, ash, length, diameter, Seedor index, and breaking strength are presented in Tables 4, 7, 8, and 11. Temperature × diet interaction was observed for

Table 9. Serum mineral composition of broiler chickens on day 28 (mg/dL)

Treatments		P	Na	Mg	K	Zn
Temperature	NT[1]	6.71	390[a]	2.14	30.12[a]	0.143
	WT[2]	6.62	385[b]	2.08	27.22[b]	0.138
Diet	SP[3]	6.33[a]	388	2.01[a]	27.81	0.135[a]
	RP[4]	7.08[b]	388	2.18[b]	27.59	0.139[ab]
	RP-Arg[5]	6.58[ab]	386	2.07[ab]	28.34	0.151[b]
	RP-Cit[6]	6.67[ab]	388	2.18[b]	30.94	0.137[ab]
Pooled SEM		0.09	0.94	0.02	0.67	0.002
p-value	Temperature	0.658	0.035	0.234	0.028	0.260
	Diet	0.035	0.857	0.016	0.264	0.042
	Temperature × diet	0.909	0.834	0.955	0.19	0.209

Note: [a,b]Differing superscripts within a column indicate significant differences between means. [1]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 24°C from days 21 to 35. [2]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 33±1 °C for 6 h per day from days 21 to 35. [3]Diet contained standard crude protein levels at 22.3% and 20.9% for grower and finisher phases, respectively. [4]Diet contained reduced crude protein levels at 19.5% and 18.4% for grower and finisher phases, respectively. [5]Reduced-protein diet supplemented with 0.29% and 0.28% L-arginine in grower and finisher phases, respectively. [6]Reduced-protein diet supplemented with 0.29% and 0.28% L-citrulline in grower and finisher phases, respectively.

Table 10. Tibia mineral composition of broiler chickens on day 28

Treatments		Ca (mg/g)	P (mg/g)	Na (mg/g)	Mg (mg/g)	K (mg/g)	S (mg/g)	Al (mg/kg)	B (mg/kg)	Cu (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Mo (mg/kg)
Temperature	NT[1]	392[a]	180[a]	13.75	8.28	7.80	3.00	78.48[a]	4.87[a]	0.545[a]	344	478	37.70[a]
	WT[2]	385[b]	178[b]	13.53	8.14	7.63	2.98	17.78[b]	2.88[b]	0.000[b]	335	470	33.89[b]
Diet	SP[3]	391	180	13.69	8.25	7.72	2.98	61.14	5.67[b]	0.417	342	479[ab]	36.96
	RP[4]	387	179	13.73	8.34	7.62	3.04	35.27	3.14[a]	0.007	323	499[b]	34.70
	RP-Arg[5]	389	179	13.52	8.09	7.71	2.88	52.45	3.63[a]	0.203	344	461[a]	36.66
	RP-Cit[6]	388	178	13.62	8.17	7.83	3.05	43.65	3.06[a]	0.463	347	457[a]	34.85
Pooled SEM		1.02	0.44	0.08	0.05	0.07	0.05	6.82	0.26	0.11	4.88	4.42	0.65
p-value	Temperature	0.001	0.001	0.142	0.139	0.284	0.835	< 0.001	< 0.001	< 0.001	0.55	0.325	0.002
	Diet	0.618	0.814	0.801	0.257	0.792	0.531	0.754	< 0.001	0.445	0.279	0.001	0.486
	Temperature × diet	0.152	0.492	0.701	0.616	0.216	0.948	0.333	0.43	0.365	0.287	0.864	0.435

Note: [a,b]Differing superscripts within a column indicate significant differences between means. [1]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 24 °C from days 21 to 35. [2]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 33±1 °C for 6 h per day from days 21 to 35. [3]Diet contained standard crude protein levels at 22.3% and 20.9% for grower and finisher phases, respectively. [4]Diet contained reduced crude protein levels at 19.5% and 18.4% for grower and finisher phases, respectively. [5]Reduced-protein diet supplemented with 0.29% and 0.28% L-arginine in grower and finisher phases, respectively. [6]Reduced-protein diet supplemented with 0.29% and 0.28% L-citrulline in grower and finisher phases, respectively.

Table 11. Tibia weight, ash, length, diameter, and breaking strength of broiler chickens on day 35

Treatments		Variables					
		Absolute weight[7] (g)	Relative weight[8] (%)	Ash[9] (%)	Length[10] (mm)	Seedor index[11] (mg/mm)	Breaking strength (N)
Temperature	NT[1]	11.034[a]	0.52	43.15	89.99[a]	59.91[a]	412
	WT[2]	10.202[b]	0.52	42.90	88.39[b]	56.67[b]	381
Diet	SP[3]	11.08	0.52	44.05[b]	89.32	59.92	409
	RP[4]	10.20	0.53	41.76[a]	89.50	57.33	364
	RP-Arg[5]	10.54	0.52	43.02[ab]	88.82	58.47	406
	RP-Cit[6]	10.64	0.52	43.26[b]	89.12	57.45	407
Pooled SEM		0.14	0.00	0.21	0.29	0.55	7.95
p-value	Temperature	0.002	0.98	0.541	0.004	0.002	0.053
	Diet	0.156	0.805	< 0.001	0.862	0.327	0.13
	Temperature × diet	0.435	0.887	0.336	0.670	0.499	0.843

Note: [a,b]Differing superscripts within a column indicate significant differences between means. [1]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 24 °C from days 21 to 35. [2]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 33±1 °C for 6 h per day from days 21 to 35. [3]Diet contained standard crude protein levels at 22.3% and 20.9% for grower and finisher phases, respectively. [4]Diet contained reduced crude protein levels at 19.5% and 18.4% for grower and finisher phases, respectively. [5]Reduced-protein diet supplemented with 0.29% and 0.28% L-arginine in grower and finisher phases, respectively. [6]Reduced-protein diet supplemented with 0.29% and 0.28% L-citrulline in grower and finisher phases, respectively. [7]Tibia weight was measured on the fresh tibia. [8]Relative tibia weight as a percentage of body weight. [9]Tibia ash was expressed as a percentage of the oven-dry tibia (%). [10]Tibia length, diameter, and breaking strength were measured on air-dry tibias. [11]Seedor index= weight of oven-dry tibia/tibia length (mg/mm).

Table 12. Serum mineral composition of broiler chickens on day 35 (mg/dL)

Treatments		Variables					
		Ca	P	Na	Mg	K	Zn
Temperature	NT[1]	9.31	6.51	395[a]	2.052	28.48	0.148
	WT[2]	9.49	6.51	385[b]	2.063	29.08	0.152
Diet	SP[3]	8.60[a]	6.25	390	1.966[a]	28.48	0.154
	RP[4]	10.16[c]	6.55	389	2.077[ab]	28.44	0.144
	RP-Arg[5]	9.47[bc]	6.51	392	2.086[ab]	28.40	0.151
	RP-Cit[6]	9.36[b]	6.72	390	2.093[b]	29.84	0.151
Pooled SEM		0.12	0.06	1.36	0.02	0.45	0.002
p-value	Temperature	0.469	0.853	< 0.001	0.76	0.509	0.226
	Diet	< 0.001	0.063	0.902	0.034	0.638	0.246
	Temperature × diet	0.935	0.301	0.796	0.13	0.494	0.806

Note: [a,b]Differing superscripts within a column indicate significant differences between means. [1]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 24 °C from days 21 to 35. [2]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 33±1 °C for 6 h per day from days 21 to 35. [3]Diet contained standard crude protein levels at 22.3% and 20.9% for grower and finisher phases, respectively. [4]Diet contained reduced crude protein levels at 19.5% and 18.4% for grower and finisher phases, respectively. [5]Reduced-protein diet supplemented with 0.29% and 0.28% L-arginine in grower and finisher phases, respectively. [6]Reduced-protein diet supplemented with 0.29% and 0.28% L-citrulline in grower and finisher phases, respectively.

tibia diameter on day 35 ($p<0.05$, Table 7), indicating that tibia diameter increased in birds fed the RP and RP-Cit diets compared to those offered the SP diet only when raised under cyclic WT. A marginal interaction between diet and room temperature was also observed for tibia ash on day 28 ($p=0.083$, Table 7), where tibia ash tended to increase in birds fed the RP-Arg compared to the RP only when raised under NT. Cyclic WT as a main effect tended to decrease absolute tibia weight ($p=0.093$) on day 28 and had more pronounced effects on tibia weight and other bone traits on day 35. Specifically, lower absolute tibia weight, length, diameter, and Seedor index were observed in birds raised under cyclic WT compared to those raised under NT on day 35 regardless of the dietary treatment ($p<0.01$, Tables 7 and 11). Also, cyclic WT birds tended to have lower tibia breaking strength compared to the NT birds regardless of the dietary treatment on day 35 ($p=0.053$, Table 11).

On day 21, birds fed the RP diet had greater relative tibia weight (as percentages of body weight, $p<0.05$), and tended to have lower tibia diameter ($p=0.051$) than those offered the SP diet (Table 4). Supplementation of Arg to the RP diet increased tibia diameter to the level of birds offered the SP diet on day 21 (Table 4). On day 28, birds fed the RP diet had lower tibia breaking strength ($p<0.05$), and tended to have lower tibia diameter ($p=0.054$) compared to those fed the SP diet, as shown by the main effect of the temperature in Table 8. Supplementation of either Arg or Cit to the RP diet increased tibia breaking strength and diameter to the levels of those offered the SP diet on day 28 (Table 8). On day 35, birds fed the RP diet had lower tibia ash

Table 13. Tibia mineral composition of broiler chickens on day 35

Treatments		Ca (mg/g)	P (mg/g)	Na (mg/g)	Mg (mg/g)	K (mg/g)	S (mg/g)	Al (mg/kg)	B (mg/kg)	Cu (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Mo (mg/kg)
Temperature	NT[1]	387^{a}	179^{a}	13.52	8.01	6.94	2.82	1.06^{a}	0.00^{a}	0.025	320	435^{a}	10.57	44.28^{a}
	WT[2]	395^{b}	182^{b}	13.66	8.12	6.93	2.79	47.35^{b}	1.76^{b}	0.018	332	456^{b}	10.14	42.07^{b}
Diet	SP[3]	390	180	13.68^{ab}	8.15	7.05^{ab}	2.83	10.27	0.53	0.000	334	455^{ab}	9.28^{a}	44.74
	RP[4]	392	180	13.39^{a}	8.11	6.80^{ab}	2.81	40.63	1.50	0.074	314	464^{b}	10.13^{ab}	42.58
	RP-Arg[5]	390	179	13.43^{ab}	7.94	6.69^{a}	2.72	22.32	0.75	0.011	317	426^{a}	10.77^{b}	42.36
	RP-Cit[6]	392	181	13.86^{b}	8.06	7.20^{b}	2.86	23.61	0.74	0.000	341	438^{ab}	11.25^{b}	43.02
Pooled SEM		0.95	0.40	0.07	0.05	0.06	0.05	6.82	0.27	0.010	4.69	4.53	0.21	0.45
p-value	Temperature	< 0.001	< 0.001	0.291	0.259	0.918	0.791	0.036	0.001	1.000	0.208	0.020	0.303	0.012
	Diet	0.852	0.63	0.044	0.432	0.01	0.719	0.538	0.536	0.233	0.116	0.010	0.003	0.228
	Temperature × diet	0.984	0.849	0.602	0.780	0.417	0.336	0.366	0.522	0.921	0.696	0.874	0.933	0.725

Note: [a,b]Differing superscripts within a column indicate significant differences between means. [1]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 24 °C from days 21 to 35. [2]Birds were raised at temperature following Ross 308 recommendation from days 0 to 21 and 33±1 °C for 6 h per day from days 21 to 35. [3]Diet contained standard crude protein levels at 22.3% and 20.9% for grower and finisher phases, respectively. [4]Diet contained reduced crude protein levels at 19.5% and 18.4% for grower and finisher phases, respectively. [5]Reduced-protein diet supplemented with 0.29% and 0.28% L-arginine in grower and finisher phases, respectively. [6]Reduced-protein diet supplemented with 0.29% and 0.28% L-citrulline in grower and finisher phases, respectively.

compared to those fed the SP diet, as shown by the main effect of the dietary treatment in Table 11 (p<0.001). Supplementation of Cit to the RP diet increased tibia ash in the respective group to the level of birds offered the SP diet on day 35 (p<0.001, Table 11).

DISCUSSION

Dietary protein level can influence bone morphology and mineralization, as it constitutes an important component of the organic structural bone matrix that may affect Ca absorption and excretion (Heaney & Layman, 2008). Dietary Arg level also appears to have pronounced effects on bone development through the process of bone mineralisation and mineral metabolism (Silva *et al.*, 2012; Castro *et al.*, 2019b). In the present study, supplementation of Cit to the RP diet increased tibia Mn level compared to the SP when birds were raised under cyclic WT on day 28. Meanwhile, supplementation of Arg to the RP diet tended to increase tibia ash compared to the RP only when birds were housed under NT and tended to increase serum Ca level compared to the SP only when housed under cyclic WT on day 28. Also, birds fed the RP diet deficient in Arg had lower tibia ash, diameter, and breaking strength compared to birds offered the SP diet and supplementation of Arg or Cit compensated for the adverse effects of the Arg-deficient RP diet on these tibia parameters. Thus, it is likely that the negative effects on tibia morphology and strength were caused by Arg deficiency or AA imbalance. Supplementation with Arg or Cit restored bone parameters. Therefore, it is crucial that reduced-protein diets maintain Arg level via Arg or Cit supplementation to avoid skeletal health issues.

Limited information is available regarding the effects of reducing dietary protein levels on blood and/or bone mineral composition in birds. In the current study, lower serum K levels in birds fed the RP diet might be attributed to the lower inclusion of soybean meal in the RP compared to the SP diet, as soybean meal is a rich source of K. The analysed mineral levels in the mixed feed confirm a lower K level for RP diets compared to SP diets in the current study. Cowieson *et al.* (2020) observed increased plasma Ca level as dietary CP level decreased (from 21.5% and 19.5% to 17.5% and 15.5% in grower and finisher phases, respectively). The present study results were in line with those previously documented in the literature. It is possible that feeding conventional CP diets affects renal function and results in a hypo-calciuretic effect that decreases Ca reabsorption and then blood Ca level (Cowieson *et al.*, 2020). Feeding conventional CP diets increases the degradation of excess sulphur AA and the resultant urinary excretion of acid and sulphate with a subsequent effect on decreasing fractional renal tubular Ca reabsorption (Zemel, 1988). Nevertheless, the higher serum P level in birds fed the RP diet compared to those offered the SP diet on day 28 in the present study might be associated with a lower Ca to P ratio in the RP diet (1.44) compared to the SP diet (1.46) during the finisher phase (days 21 to 35). It is known that reducing the dietary Ca to P ratio increases P absorption due to increased luminal Ca active transport in response to the increased vitamin D secretion (Lips, 2012). Finally, the decreases in serum Ca, P, and Mg levels following Arg or Cit supplementation to the RP diet in the current study suggest that elevated levels of these minerals in the blood serum of birds fed the RP diet might be associated with Arg deficiency and/or AA imbalance.

The findings of the current study indicated that birds fed the RP diet had lower tibia B and tended to have lower tibia Fe levels, but also had higher tibia Mn levels compared to those offered the SP diet on day 21. Additionally, birds fed the RP diet exhibited lower tibia B levels compared to those offered the SP diet on day 28. Lower bone mineral density has been reported in broilers fed a diet deficient in Arg compared to those fed a diet with sufficient Arg level (Castro *et al.*, 2019b). A recent study by Cowieson *et al.* (2020) revealed that tibia Mn and Cu levels decreased as dietary protein levels decreased (from 21.5% and 19.5% to 17.5% and 15.5% in the grower and finisher phase, respectively). A strong correlation between dietary B level and B levels in the plasma and tibia was observed by Kurtoğlu *et al.* (2005). The authors also reported that dietary B supplementation resulted in increased plasma Fe levels (Kurtoğlu *et al.*, 2005). Thus, the lower levels of tibia B and Fe observed in birds fed the RP diet in the current study might be attributed to a lower dietary B level in the RP compared to the SP diet. Increased tibia ash, Ca, and P levels in laying quails during the late laying phase following the dietary supplementation of Arg silicate inositol complex (49.5% arginine, 8.2% silicon, and 25% inositol) have been reported and speculated to associate with the increases in Ca and P availability in their respective groups (Onderci *et al.*, 2006). In the current study, supplementation of either Arg or Cit to the RP diet decreased tibia Zn level compared to the RP on day 28, and increased tibia Mn level compared to the SP on day 35. Supplementation of Arg to the RP diet decreased tibia Zn level compared to the RP and decreased tibia K level compared to the RP-Cit on day 35. Whereas supplementation of Cit to the RP diet increased tibia Na level compared to the RP on day 35. These results reflect the differential effects of Arg and Cit supplementation to the RP diet on tibia mineral composition. Evaluation of micro-mineral status remains an important but complex challenge (Hosseini & Afshar, 2017). Further work is necessary to determine the mechanism underpinning the bone mineralisation effects of Arg and Cit supplementation to RP diets for broilers.

Heat stress has been reported to reduce bone weight, ash content, length, width, and breaking strength in broiler chickens (Sgavioli *et al.*, 2016; Mosleh *et al.*, 2018), which was observed in the present study. Additionally, the present study indicated that the effects of heat stress on bone parameters might depend on its duration. Cyclic WT tended to decrease tibia weight in the first week but had more pronounced effects on tibia weight and other bone parameters (length, diameter, Seedor index, and tibia breaking strength) in the second week. Heat stress may compromise body electrolyte balance resulting in reduced bone development and increased leg problems (Patience, 1990; Luo *et al.*, 2018). Consequently, fast-growing broilers raised under high temperatures typically suffer considerable skeletal problems and lameness (Abioja *et al.*, 2012). Although the level of Cl^- was not measured in the current study, decreased serum Na and K levels on day 28, and decreased serum Na level on day 35 was observed in cyclic WT birds compared to the NT birds. The reduced serum Na and K levels in heat-exposed birds may be attributed to the impaired absorption/retention of these minerals and/or haem-dilution status induced by altered membrane permeability in the respective group (Khattak *et al.*, 2012). It has been observed that the addition of a mineral-vitamin mix consisting of sodium bicarbonate (49.5%), potassium chloride (37.5%), magnesium acetate (3.0%), and vitamin C (8.9%) in drinking water increased feed intake, weight gain, and feed efficiency in birds raised under warm temperatures (del Barrio *et al.*, 2020). Likewise, the bone mineral level might alter during the heat stress due to the decrease of mineral intake and increase of mineral excretion resulting from reduced feed consumption and absorption in heat-exposed birds (Farag & Alagawany, 2018). Sgavioli *et al.* (2016) indicated that tibia mineral level, mineral density, and Ca level decreased in broilers raised under high temperatures (32±7 °C from days 0 to 42) compared to those raised under NT on day 42. Similarly, lower tibia ash, Ca, P, Mg, and Mn levels were observed in laying quails during the heat stress (34 °C for 8 h per day, Onderci *et al.*, 2006). In the current study, cyclic WT as the main effect reduced tibia Ca, P, Al, B, Cu, Mo, and Mn levels on day 28. Surprisingly, lower tibia Mo levels but higher tibia Ca, P, Al, B, and Zn levels were observed in cyclic WT birds compared to the NT birds on day 35. Metabolic acidosis may occur in birds during the heat stress that first stimulates mineral degradation, then the cell-mediated bone resorption due to increased Ca excretion in the kidney (Oliveira *et al.*, 2010). When birds are raised under stress conditions, mineral requirements are prioritised for vital functions rather than growth (Hosseini & Afshar, 2017). Birds might increase tibia mineral deposition as an adaptation mechanism to fight against heat stress. It is also worth noting that only moderate temperature was applied in the cyclic WT room in the current study (33±1 °C for 6 h per day).

CONCLUSION

In conclusion, supplementation of Arg or Cit to the RP diet reduced the adverse effects of Arg-deficient RP diet and/or cyclic WT on serum minerals, tibia morphology, and tibia mineral composition by increasing serum K level, tibia diameter, breaking strength, total ash, Na and Mn levels.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

ACKNOWLEDGEMENT

The authors would like to thank Poultry Hub Australia for their financial support for this study.

REFERENCES

Abioja, M. O., K. B. Ogundimu, T. E. Akibo, K. E. Odukoya, O. O. Ajiboye, J. A. Abiona, T. J. Williams, E. O. Oke, & O. A. Osinowo. 2012. Growth, mineral deposition, and physiological responses of broiler chickens offered honey in drinking water during hot-dry season. Int. J. Zool. 2012: Article ID 403502. https://doi.org/10.1155/2012/403502

Attia, Y. A., R. A. Hassan, A. E. Tag El-Din, & B. M. Abou-Shehema. 2011. Effect of ascorbic acid or increasing metabolizable energy level with or without supplementation of some essential amino acids on productive and physiological traits of slow-growing chicks exposed to chronic heat stress. J. Anim. Physiol. Anim. Nutr. 95:744-755. https://doi.org/10.1111/j.1439-0396.2010.01104.x

Aviagen. 2014a. Ross 308 Broiler Management Handbook. Ross Breeders Limited, Newbridge, Midlothian, Scotland, UK.

Aviagen. 2014b. Ross 308 Broiler Nutrition Specification. Ross Breeders Limited, Newbridge, Midlothian, Scotland, UK.

Belhadj, S. I., T. Najar, A. Ghram, & M. Abdrrabba. 2016. Heat stress effects on livestock: molecular, cellular and metabolic aspects, a review. J. Anim. Physiol. Anim. Nutr. 100:401-412. https://doi.org/10.1111/jpn.12379

Belloir, P., B. Méda, W. Lambert, E. Corrent, H. Juin, M. Lessire, & S. Tesseraud. 2017. Reducing the CP content in broiler feeds: impact on animal performance, meat quality and nitrogen utilization. Animals. 11:1881-1889. https://doi.org/10.1017/S1751731117000660

Brickett, K. E., J. P. Dahiya, H. L. Classen, C. B. Annett, & S. Gomis. 2007. The impact of nutrient density, feed form, and photoperiod on the walking ability and skeletal quality of broiler chickens. Poult. Sci. 86:2117-2125. https://doi.org/10.1093/ps/86.10.2117

Castro, F. L. S., H. Y. Kim, Y. G. Hong, & W. K. Kim. 2019a. 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. https://doi.org/10.3382/ps/pez275

Castro, F. L. S., S. Su, H. Choi, E. Koo, & W. K. Kim. 2019b. L-arginine supplementation enhances growth performance, lean muscle, and bone density but not fat in broiler chickens. Poult. Sci. 98:1716-1722. https://doi.org/10.3382/ps/pey504

Chrystal, P. V., A. F. Moss, A. Khoddami, V. D. Naranjo, P. H. Selle, & S. Y. Liu. 2020. Effects of reduced crude protein levels, dietary electrolyte balance, and energy density on the performance of broiler chickens offered maize-based diets with evaluations of starch, protein, and amino acid metabolism. Poult. Sci. 99:1421-1431. https://doi.org/10.1016/j.psj.2019.10.060

Cowieson, A. J., R. Perez-Maldonado, A. Kumar, & M. Toghyani. 2020. Possible role of available phosphorus in potentiating the use of low protein diets for broiler chicken production. Poult. Sci. 99:6954-6963. https://doi.org/10.1016/j.psj.2020.09.045

Dao, H. T., N. K. Sharma, E. J. Bradbury, & R. A. Swick. 2021a. Response of meat chickens to different sources of arginine in low-protein diets. J. Anim. Physiol. Anim. Nutr. 105:731-746. https://doi.org/10.1111/jpn.13486

Dao, H. T., N. K. Sharma, E. J. Bradbury, & R. A. Swick. 2021b. Effects of L-arginine and L-citrulline supplementation in reduced protein diets for broilers under normal and cyclic warm temperature. Anim. Nutr. 7:927-938. https://doi.org/10.1016/j.aninu.2020.12.010

Dao, H. T., N. K. Sharma, E. J. Bradbury, & R. A. Swick. 2021c. Response of laying hens to L-arginine, L-citrulline and guanidinoacetic acid supplementation in reduced protein diet. Anim. Nutr. 7:460-471. https://doi.org/10.1016/j.aninu.2020.09.004

del Barrio, A. S., W. D. Mansilla, A. Navarro-Villa, J. H. Mica, J. H. Smeets, L. A. den Hartog, & A. I. García-Ruiz. 2020. Effect of mineral and vitamin C mix on growth performance and blood corticosterone concentrations in heat-stressed broilers. J. Appl. Poult. Res. 29:23-33. https://doi.org/10.1016/j.japr.2019.11.001

Esser, A. F. G., D. R. M. Gonçalves, A. Rorig, A. B. Cristo, R. Perini, & J. I. M. Fernandes. 2017. Effects of guanidionoacetic acid and arginine supplementation to vegetable diets fed to broiler chickens subjected to heat stress before slaughter. Rev. Bras. Cienc. Avic. 19:429-436. https://doi.org/10.1590/1806-9061-2016-0392

Farag, M. R. & M. Alagawany. 2018. Physiological alterations of poultry to the high environmental temperature. J. Therm. Biol. 76:101-106. https://doi.org/10.1016/j.jtherbio.2018.07.012

Fouad, A. M., H. K. El-Senousey, X. J. Yang, & J. H. Yao. 2013. Dietary L-arginine supplementation reduces abdominal fat content by modulating lipid metabolism in broiler chickens. Animals. 7:1239-1245. https://doi.org/10.1017/S1751731113000347

Heaney, R. P. & D. K. Layman. 2008. Amount and type of protein influences bone health. Am. J. Clin. Nutr. 87:1567S-1570S. https://doi.org/10.1093/ajcn/87.5.1567S

Hilliar, M., N. Huyen, C. K. Girish, R. Barekatain, S. Wu, & R. A. Swick. 2019. Supplementing glycine, serine, and threonine in low protein diets for meat type chickens. Poult. Sci. 98:6857-6865. https://doi.org/10.3382/ps/pez435

Hosseini, S. M. & M. Afshar. 2017. Effect of diet form and enzyme supplementation on stress indicators and bone mineralisation in heat-challenged broilers fed wheat-soybean diet. Ital. J. Anim. Sci. 16:616-623. https://doi.org/10.1080/1828051X.2017.1321973

Jahanian, R. 2009. Immunological responses as affected by dietary protein and arginine concentrations in starting broiler chicks. Poult. Sci. 88:1818-1824. https://doi.org/10.3382/ps.2008-00386

Khattak, F. M., T. Acamovic, N. Sparks, T. N. Pasha, M. H. Joiya, Z. Hayat, & Z. Ali. 2012. Comparative efficacy of different supplements used to reduce heat stress in broilers. Pak. J. Zool. 44:31-41.

Kurtoğlu, F., V. Kurtoğlu, I. Celik, T. Kececi, & M. Nizamlioğlu. 2005. Effects of dietary boron supplementation on some biochemical parameters, peripheral blood lymphocytes, splenic plasma cells and bone characteristics of broiler chicks given diets with adequate or inadequate cholecalciferol (vitamin D3) content. Br. Poult. Sci. 46:87-96. https://doi.org/10.1080/00071660400024001

Kvidera, S. K., E. A. Horst, E. J. Mayorga, J. T. Seibert, M. A. Al-Qaisi, J. W. Ross, R. P. Rhoads, & L. H. Baumgard. 2016. 0995 Effect of supplemental citrulline on thermal and production parameters during heat stress in growing pigs. J. Anim. Sci. 94:477. https://doi.org/10.2527/jam2016-0995

Lips, P. 2012. Interaction between vitamin d and calcium. Scand. J. Clin. Lab. Invest. 72:60-64.

Liu, F., E. M. de Ruyter, R. Z. Athorn, C. J. Brewster, D. J. Henman, R. S. Morrison, R. J. Smits, J. J. Cottrell, & F. R. Dunshea. 2019. Effects of L-citrulline supplementation on heat stress physiology, lactation performance and subsequent reproductive performance of sows in summer. J. Anim. Physiol. Anim. Nutr. 103:251-257. https://doi.org/10.1111/jpn.13028

Luo, J., J. Song, L. Liu, B. Xue, G. Tian, & Y. Yang. 2018. Effect of epigallocatechin gallate on growth performance and serum biochemical metabolites in heat-stressed broilers. Poult. Sci. 97:599-606. https://doi.org/10.3382/ps/pex353

Mosleh, N., T. Shomali, F. Nematollahi, Z. Ghahramani, M. S. A. Khadi, & F. Namazi. 2018. Effect of different periods of chronic heat stress with or without vitamin C supplementation on bone and selected serum parameters of broiler chickens. Avian Pathol. 47:197-205. https://doi.org/10.1080/03079457.2017.1401212

NHMRC. 2013. Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. 8th Ed. The National Health and Medical Research Council, Australia.

Oliveira, M. C., U. M. Arantes, & J. H. Stringuini. 2010. Efeito do balanco eletrolitico da racao sobre parametros osseos e da cama de frango. Biotemas. 23:203-209. https://doi.org/10.5007/2175-7925.2010v23n1p203

Onderci, M., N. Sahin, K. Sahin, T. A. Balci, M. F. Gursu, V. Juturu, & O. Kucuk. 2006. Dietary arginine silicate inositol complex during the late laying period of quail at different environmental temperatures. Br. Poult. Sci. 47:209-215. https://doi.org/10.1080/00071660600611052

Patience, J. F. 1990. A review of the role of acid-base balance in amino acid nutrition. J. Anim. Sci. 68:398-408. https://doi.org/10.2527/1990.682398x

Seedor, J. G., H. A. Quartuccio, & D. D. Thompson. 1991. The biophosphonate alendronate (MK - 217) inhibits bone loss due to ovariectomy in rats. J. Bone Miner. Res. 6:339-346. https://doi.org/10.1002/jbmr.5650060405

Sgavioli, S. C. **H. F. Domingues, E. T. Santos, T. C. O. de Quadros, L. L. Borges, R. G. Garcia, M. J. Q. L. Louzada, & I. C. Boleli.** 2016. Effect of *in-ovo* ascorbic acid injection on the bone development of broiler chickens submitted to heat stress during incubation and rearing. Rev. Bras. Cienc. Avic. 18:153-162. https://doi.org/10.1590/18069061-2015-0075

Silva, L. M. G. S., A. E. Murakami, J. I. M. Fernandes, D. Dalla Rosa, & J. F. Urgnani. 2012. Effects of dietary arginine supplementation on broiler breeder egg production and hatchability. Rev. Bras. Cienc. Avic. 14:267-273. https://doi.org/10.1590/S1516-635X2012000400006

Su, C. L. & R. E. Austic. 1999. The recycling of L-citrulline to L-arginine in a chicken macrophage cell line. Poult. Sci. 78:353-355. https://doi.org/10.1093/ps/78.3.353

Talaty, P. N., M. N. Katanbaf, & P. Y. Hester. 2009. Life cycle changes in bone mineralisation and bone size traits of commercial broilers. Poult. Sci. 88:1070-1077. https://doi.org/10.3382/ps.2008-00418

Wu, S. B., R. A. Swick, J. Noblet, N. Rodgers, D. Cadogan, & M. Choct. 2019. Net energy prediction and energy efficiency of feed for broiler chickens. Poult. Sci. 98:1222-1234. https://doi.org/10.3382/ps/pey442

Zaman, Q. U., T. Mushtaq, H. Nawaz, M. A. Mirza, S. Mahmood, T. Ahmad, M. E. Babar, & M. M. H. Mushtaq. 2008. Effect of varying dietary energy and protein on broiler performance in hot climate. Anim. Feed Sci. Technol. 146:302-312. https://doi.org/10.1016/j.anifeedsci.2008.01.006

Zanu, H. K., S. K. Kheravii, N. K. Morgan, M. R. Bedford, & R. A. Swick. 2020. Interactive effect of dietary calcium and phytase on broilers challenged with subclinical necrotic enteritis: 3. serum calcium and phosphorus, and bone mineralization. Poult. Sci. 99:3617-3627. https://doi.org/10.1016/j.psj.2020.04.012

Zemel, M. B. 1988. Calcium utilization: effect of varying level and source of dietary protein. Am. J. Clin. Nutr. 48:880-883. https://doi.org/10.1093/ajcn/48.3.880