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Original Research Article

Effects of L-arginine and L-citrulline supplementation in reduced protein diets for broilers under normal and cyclic warm temperature

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ABSTRACT

Heat stress causes significant economic losses in the broiler industry. Dietary supplementation of arginine (Arg) and citrulline (Cit) might increase the performance of broilers raised under warm temperature due to vasodilation effects. This study investigated the effects of L-Arg or L-Cit supplementation in broilers fed a reduced protein wheat-based diet deficient in Arg under thermoneutral (NT) and cyclic warm temperature (WT). Ross 308 cockerels ($n = 720$) were randomly allocated to 4 dietary treatments with 12 replicates of 15 birds per pen from d 7 to 21. The 4 treatments were: normal protein (NP), i.e., 22.3% and 20.9% crude protein in grower and finisher, respectively; reduced protein (RP), i.e., 2.5% lower protein and deficient in Arg; and RP supplemented with 0.28% Arg (RP-Arg) or 0.28% Cit (RP-Cit). A factorial arrangement of treatments was applied during the finisher phase (21 to 35 d). Factors were: diet (4 diets above); and temperature, NT (24 °C) or cyclic WT (33 ± 1 °C for 6 h per day) with 6 replicate pens per treatment. During 7 to 35 d and 21 to 35 d, the birds fed the RP diet had lower body weight gain (BWG) and higher FCR compared to the NP diet ($P < 0.01$). The addition of Arg or Cit to RP decreased FCR compared to RP ($P < 0.01$). During 21 to 35 d, the birds exposed to WT had lower feed intake (FI), lower BWG ($P < 0.001$) but similar FCR ($P > 0.05$) compared to birds exposed to NT. Diet by temperature interactions were not observed for performance parameters during the period of WT ($P > 0.05$). On d 35, the RP-fed birds had a lower yield of thigh and drumstick, higher fat pad, lower femur ash, and breaking strength but similar serum uric acid level and higher nitrogen digestibility on d 21 compared to those offered NP ($P < 0.05$). Supplementation of Arg or Cit to RP resulted in increased femur ash on d 35 ($P < 0.05$). Thus, feeding the NP diets is necessary to maintain growth performance in broilers regardless of the temperature conditions.

1. Introduction

Heat stress is a common issue in the broiler industry and can result in significant economic losses (Yatoo et al., 2012) and compromised welfare (Castro et al., 2019). It has been reported that heat stress alters physiological response, reduces nutrient digestion, impairs intestinal barrier integrity, decreases growth performance, and often leads to high mortality in birds (Lara and Rostagno, 2013; Lu et al., 2017; Sergio et al., 2018; Song et al., 2018; He et al., 2018ab). Also, heat stress may compromise body electrolyte balance and reduce calcium absorption, affecting bone development and mineralization resulting in the increased incidence of leg problems in young broilers (Patience, 1990; Gonzalez-Esquerra and Leeson, 2006).

Arginine (Arg) is an essential amino acid (AA) for poultry and plays an important role in various metabolic pathways including protein synthesis and immunity. Arginine is converted to nitric oxide at the macrophage level (Jahanian, 2009). Nitric oxide has been recognized as a mediating factor for vasodilation and increased peripheral blood flow that is an important thermoregulatory response

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to heat stress (Liu et al., 2019). Chickens have low activities of key enzymes involved in endogenous Arg synthesis (Sung et al., 1991); thus, they have minimal Arg de novo synthesis and are strongly dependent on dietary sources for Arg. Dietary supplementation of Arg has been reported to have positive effects on growth performance, carcass yield, gut morphology, bone shape and quality in broilers under thermoneutral temperature (Corzo and Kidd, 2003a; Dilger et al., 2013; Castro et al., 2018; Abdulkarimi et al., 2019). Furthermore, Arg has been demonstrated to effectively compensate for reduced growth performance in birds (Balnave and Brake, 2002) and body weight loss in lactating sows subjected to heat stress (Laspiur and Trottier, 2001).

Protein metabolism is associated with higher heat production compared to fat and carbohydrate metabolism (Musharaf and Latshaw, 1999; Wu et al., 2019). Hence, reduction of dietary CP as a means to limit heat production especially during heat stress has been recommended by some (Waldroup et al., 1976; Teeter, 1996). It is known that crystalline AA are more digestible and more bioavailable than protein-bound amino acids in the form of intact protein (Chung and Baker, 1991; Hilliar et al., 2019). With higher rates of crystalline AA and lower rates of protein-bound amino acids in reduced CP diets, birds use dietary nitrogen more efficiently and produce less uric acid (Hilliar et al., 2019) being a potential benefit in birds exposed to high ambient temperatures. Citrulline (Cit) is a non-protein AA and metabolite of Arg that is co-produced with nitric oxide by macrophages and can be recycled to Arg. Studies in humans and mammals have indicated that Cit is more effective than dietary Arg in increasing blood Arg levels (Schwedhelm et al., 2008; Lassala et al., 2009) and nitric oxide production (Wijnands et al., 2012). In broilers, Cit has been shown to have Arg-sparing effects (Klose and Almquist, 1940; Tamir and Ratner, 1963; Su and Austic, 1999); however, information on the effects of Cit supplementation on growth performance and other parameters is extremely limited. A recent study by Dao et al. (2020) indicated that supplementation of Cit to an Arg-deficient reduced protein diet for broilers resulted in similar growth performance, carcass, and bone traits compared to those fed the Arg-deficient reduced protein diet supplemented with Arg. Citrulline supplementation has been shown to reduce the respiration rate and lower piglet pre-weaning mortality rate under hot conditions (Kvidera et al., 2016; Liu et al., 2019). The present study was designed to investigate the effect of protein level, deficiency of Arg, and supplementation of crystalline Arg and Cit as substitutes for Arg in broilers raised under thermoneutral (NT) or cyclic warm temperature (WT). The parameters of interest measured were growth performance, carcass traits, organ weights, ileal nutrient digestibility, serum uric acid (SUA), gut permeability, bone shape and quality. Dietary Arg:Lysine (Lys) imbalance caused by an excess of Lys (low Arg-to-Lys ratio) or Arg (high Arg-to-Lys ratio) has been reported to reduce the growth performance of birds (Balnave and Brake, 2002). Furthermore, the Arg-to-Lys ratio to support growth performance might increase at high temperatures due to the reduction of Arg uptake in the digestive tract (Balnave and Brake, 2002). Thus, it was hypothesized that feeding a reduced protein diet deficient in Arg would decrease the Arg-to-Lys ratio and thereby exacerbate the negative effects of cyclic WT on bird performance. Supplementation of either Arg or Cit to correct the Arg:Lys imbalance was hypothesized as a way to maintain performance in birds fed Arg-deficient reduced protein diets. The results of the current study will be of interest to nutritionists formulating diets for broilers growing in warm climatic conditions.

2. Materials and methods

2.1. Experimental design and diets

The study was implemented at the Centre of Animal Research and Teaching at the University of New England, Armidale, New South Wales, Australia, approved by its Animal Ethics Committee and met the requirements of the Australian code of practice to care and use of animals for scientific purposes (NHMRC, 2013). Day-old Ross 308 male birds ($n = 720$) were brooded together for the first 7 d in a temperature-controlled room and fed a common starter diet containing 3,000 kcal/kg nitrogen-corrected metabolizable energy (MEn) and 24.6% CP. On d 8, birds were weighed and randomly allocated to 48 equal-sized floor pens (120 cm × 80 cm), 15 birds per pen with wood shavings used as a bedding material. Birds were raised in 2 temperature-controlled rooms with equal numbers of replicates per treatment for each room. Starting d 7 pen weights were not different across treatments ($P > 0.05$). A completely randomized design blocked by room was used consisting of 4 dietary treatments with 12 replicates (6 per room) with a pen as an experimental unit. Feed and water were provided ad libitum throughout the study. The temperature and lighting program were set as per Ross 308 recommendation (Aviagen, 2014a) from d 0 to 21. From d 21 to 35, the lighting program continued to follow Ross 308 recommendation, whereas the temperature in one of the climate-controlled rooms was set to cycle up to 33 ± 1 °C for 6 h per day (WT) from 10:30 to 16:30 using a 1-h gradient to slowly increase and decrease the temperature. The temperature for the remaining time of the day in the warm room was the same as the NT room being thermoneutral or 24 °C. The room as the main factor was found to have no effects on growth performance and livability from d 7 to 21 ($P > 0.05$).

The diets were: normal protein (NP), reduced protein deficient in Arg (RP), RP supplemented with Arg (Arg level equivalent to NP) (RP-Arg), and RP supplemented with Cit (Cit level equivalent to Arg on a molar basis) (RP-Cit). There was approximately 2.5 percentage points difference in CP level between NP and RP treatments. The dietary energy levels were selected based on the Ross 308 broiler nutrition specifications (Aviagen, 2014b). Meanwhile, requirements of digestible AA in the diets were calculated by AMINOChick 2.0 software (Evonik Animal Nutrition) based on bird age and dietary energy level. Supplemental levels of Arg and Cit in grower and finisher diets were 0.29% and 0.28%, respectively. Details on diet composition and nutrient content are presented in Tables 1 and 2. Arginine and Cit were supplemented in RP diets at the expense of wheat. Feeds were provided as crumbles for starter (d 0 to 7), and pellets for grower (d 7 to 21) and finisher (d 21 to 35). Meat and bone meal was included in the diets to satisfy the requirement for available phosphorus. The nutritional composition of wheat, sorghum, meat and bone meal, soybean meal, and canola meal were analyzed before diet formulation. Crude protein, crude fat, dry matter, and ash content were measured using AOAC methods (AOAC, 1994) and metabolizable energy, total and digestible AA were estimated using near-infra red reflectance spectroscopy (Foss NIR 6500, Denmark) standardized with the Evonik AMINONIR Advanced calibration. Titanium dioxide was included in grower diets at 0.5% as an inert marker for digestibility determination.

2.2. Data collection

Body weight (BW) and feed consumption were measured per pen for each feeding phase. Body weight gain (BWG) and feed

Table 1
Diet composition and calculated nutrient values of normal and reduced protein diets (%, as-fed basis).

Item	Starter	Grower		Finisher	
		NP[1]	RP[2]	NP[1]	RP[2]
Ingredients					
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
MEn, kcal/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[8], mEq/kg	272	238	188	214	167

[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, 500 FTU/kg (Natuphos E 5000 G, BASF Corporation, Florham Park, NJ, US).
[4] Vitamin premix per kilogram 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 kilogram diet (UNE TM, Rabar Pty Ltd): Cu, 16 mg as copper sulfate; Mn, 60 mg as manganese sulfate; Mn, 60 mg as manganous oxide; I, 0.125 mg as potassium iodide; Se, 0.3 mg; Fe, 40 mg, as iron sulfate; Zn, 50 mg as zinc oxide; Zn, 50 mg as zinc sulfate.
[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 sulfur amino acids (methionine + cysteine).
[8] Dietary electrolyte balance calculated as 10,000 × ($Na^+ + K^+ - Cl^-$).

conversion ratio (FCR), was expressed as feed-to-gain ratio. The FCR was corrected for mortality by adding the weight of dead birds to live birds for each period. Feed intake (FI) was calculated as the corrected FCR multiplied by BWG. On d 21, 2 birds per pen were randomly collected, weighed, electrically stunned (MEFE CAT 44N, Mitchell Engineering Food Equipment, Clontarf, QLD, Australia), and euthanized by decapitation for collection of blood (from jugular vein), ileal digesta, and leg bone. Similar procedures were applied to collect blood and leg bone samples on d 28 and blood, leg bone, carcass, and internal organs on d 35. On d 21, blood and ileal digesta were collected for uric acid and apparent nutrient digestibility analyses (energy and nitrogen), respectively. Ileal digesta were obtained by gently squeezing the whole ileum (from Meckel's diverticulum to 1 cm before the ileal–cecal junction) into 50 mL-containers and were stored at −20 °C until further analysis. The fluorescein isothiocyanate dextran (FITC-d, Sigma Aldrich, New South Wales, Australia) was used as a marker to measure the gut permeability at d 28. The FITC-d solution was prepared, kept at 4 °C, and wrapped in aluminium foil to avoid light exposure. On d 28, 2 birds per pen were selected, marked, and inoculated with a 1-mL

Table 2
Analyzed nutrient values of experimental diets (%, as-fed basis).

Nutrient composition	Grower								Finisher							
	NP[1]		RP[2]		RP-Arg[3]		RP-Cit[4]		NP[1]		RP[2]		RP-Arg[3]		RP-Cit[4]	
Dry matter	86.52		86.91		86.70		86.66		86.41		86.91		86.63		86.08	
GE, kcal/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)

[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 parentheses were calculated nutrients.
[6] Values of all amino acids presented were total amino acids.
[7] Total sulfur amino acids (methionine + cysteine).

dose of FITC-d (4.17 mg/kg BW). Blood samples were collected between 2 and 2.5 h after the inoculation. On d 35, 3 birds per pen were randomly selected and blood samples were collected for uric acid assays after decapitation. Weights of different carcass cuts (breast, thigh and drumstick, abdominal fat), ileum and immunity organs (the liver, spleen, bursa of Fabricius), and ileum length were also collected. Femur samples were collected at all sampling days (d 21, 28, and 35) from the right legs. Femurs were separated and cleaned by using a knife and scissors. Initial weights of fresh femurs were recorded and dried in a fume hood for 48 h. Weights of air-dry femurs were recorded and samples were kept in a cool room at 4 °C until further analysis.

2.3. Analysis of uric acid and FITC-d levels in blood serum

Uric acid and FITC-d were measured in blood serum on d 21, d 35, and d 28, respectively. Blood samples were collected in vacutainers (Becton, Dickinson U.K. Limited, Plymouth, UK) that contained spray-coated silica and a polymer gel and centrifuged at 3,000 × *g* at 4 °C for 10 min to separate the serum. Serum samples were stored at −20 °C until further analysis. Serum uric acid level was quantified in duplicate using a URCA method (REF number: DF77, Siemens Healthcare, Newark, NJ, US) in an integrated chemistry analyzer (Siemens Dimension Xpand Plus, Siemens Healthcare, Newark, NJ, US). To determine FITC-d concentration, a standard curve was prepared following the method previously described by Prado-Rebolledo et al. (2017). The FITC-d levels of diluted serum samples (1:1 PBS), blank, and standards were determined at an excitation wavelength of 480 nm and an emission wavelength of 520 nm using a Synergy MX plate reader (Biotek Instruments, Bedfordshire, UK).

2.4. Feed and ileal digestibility analysis

The nutrient composition including CP, dry matter, crude fat, crude fiber, ash contents, and AA profiles of diets were analyzed by standard methods (AOAC, 1994). Added Arg and Cit in RP-Arg and RP-Cit diets were quantified using the Waters AccQTag amino acid analysis methodology (Cohen, 2001) but adapted to run on an ultra-performance liquid chromatography system as described by Wheat et al. (2008). Specifically, samples (100 to 130 mg) were weighed in duplicate into hydrolysis vials and 5 mL of 20% HCl was added. The samples were then incubated at 110 °C for 24 h. After hydrolysis, the samples were derivatized using AccQTag reagents (Waters Corporation, Milford, MA, US). Then, samples were analyzed using a high-resolution reversed-phase column (BEH C18, 2.1 mm × 100 mm; 1.7 μm) on an ultra-performance liquid chromatography system with 12-min run time. The column temperature, detection wavelength, and flow rate employed were 57 °C, 260 nm and 0.55 mL/min, respectively.

To analyze titanium concentration, ileal digesta samples were freeze-dried (Christ Alpha 1-4 LD plus, Osterode am Harz, Germany) and ground to a particle size of ≤0.5 mm. Ground feed and ileal digesta samples were analyzed for titanium content in duplicates following a colorimetric method previously described by Short et al. (1996). If the variation between duplicates was greater than 5%, the sample was re-analyzed. Nitrogen concentrations in feed and ileal digesta samples were measured using the Dumas combustion method (Dumas, 1831) in a nitrogen analyzer (LECO Corporation, St Joseph, MI, US) with ethylene diamine tetraacetic acid (EDTA) as a calibration standard. Gross energy levels in feed and ileal digesta samples were determined by a Parr adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL, USA)

calibrated using benzoic acid as a standard. Dry matter content of feed and freeze-dried ileal digesta were measured by oven drying the samples at 105 °C for 24 h for calculations of titanium concentration as per dry matter basis.

2.5. Measurement of bone parameters

Air-dried femur samples (48 h under the fume hood) were subjected to bone ash, length, diameter, and breaking strength measurements. Bone samples were dried in a forced-air oven at 105 °C for 24 h and ashed at 600 °C for 13 h. Bone length and diameter (at the medial region of the bone) were determined using an electronic caliper. Then bone breaking strength was measured using a Lloyd Testing Instrument (model 1000R, Lloyd Instruments Ltd., Fareham, Hampshire, UK).

2.6. Calculations and data analyses

Equations described by Jasek et al. (2018) were used to calculate apparent ileal digestible energy (IDE) and coefficients of nitrogen (IDNC) and energy digestibility (IDEC) as below.

$$IDE = GE_{diet} - \left[GE_{digesta} \times \left(\frac{Ti_{diet}}{Ti_{digesta}}\right)\right]$$

$$IDNC = 1 - \left(\frac{Ti_{diet} \times N_{digesta}}{Ti_{digesta} \times N_{diet}}\right)$$

$$IDEC = 1 - \left(\frac{Ti_{diet} \times GE_{digesta}}{Ti_{digesta} \times GE_{diet}}\right)$$

Where GE_{diet} and $GE_{digesta}$ were gross energy of the treatment diets and ileal digesta, respectively, Ti_{diet} and $Ti_{digesta}$ were titanium dioxide concentrations in the diet and ileal digesta, respectively, and N indicated either feed or ileal digesta nitrogen content.

Bone Seedor index (mg/mm) was calculated following an equation described by Seedor et al. (1991).

$$\text{Bone Seedor index}(mg/mm) = \frac{\text{Weight of oven dry bone}(mg)}{\text{Bone length}(mm)}$$

R Commander (version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria) was used to analyze data. Data were tested for normality and variance homogeneity then either one-way ANOVA or the non-parametric ANOVA (Kruskal–Wallis test) was used to test statistical differences between the treatment means (4 dietary treatments: NP, RP, RP-Arg, and RP-Cit, or temperature conditions: NT and WT). A two-way ANOVA was used to test the interaction between temperature and dietary treatments. As livability data were not normally distributed, they were tested for significance using the Kruskal–Wallis non-parametric test and were not subjected to two-way ANOVA. Tukey's post-hoc test was used to identify pairwise differences between the treatment means from significant ANOVA results. The P-value ≤ 0.05 was considered significant and a tendency was considered at $0.05 < P \leq 0.10$.

3. Results

The final diets satisfied formulation objectives in terms of achieving reduced CP diets deficient in Arg. The analyzed AA contents of the diets were comparable with the calculated values (Tables 1 and 2).

3.1. Growth performance

Results on growth performance are presented in Tables 3 and 4. There was no temperature by diet interaction on FI, BWG, and FCR during the finisher phase from 21 to 35 d ($P > 0.05$, Table 4). Birds exposed to the cyclic WT had 7.2% lower FI ($P < 0.001$), 10.6% lower BWG ($P < 0.001$), but similar FCR ($P > 0.05$) compared to those reared in NT from 21 to 35 d. Birds fed the RP diet had lower BWG and higher FCR compared to those offered the NP diet from 21 to 35 d ($P < 0.01$). The addition of Arg to RP increased BWG and was not different to birds fed the NP diet ($P > 0.05$) whereas the addition of Cit to the RP diet did not affect BWG ($P > 0.05$) from d 21 to 35. The FCR decreased when Arg or Cit was supplemented to the RP diet but the FCR in these groups were still higher than the NP group from d 21 to 35 ($P < 0.001$, Table 4). During the grower phase from d 7 to 21, the dietary treatments had no effect on FI, BWG, and livability of birds ($P > 0.05$, Table 3). The addition of Arg to the grower RP diet decreased FCR by 6 points ($P < 0.05$); however, groups fed RP, RP-Cit and NP had a similar FCR ($P > 0.05$, Table 3). Over the entire experimental period (d 7 to 35), birds fed the RP diet had lower BWG and higher FCR compared to those fed the NP diet ($P < 0.01$, Table 4). Supplementation of Arg or Cit to the RP diet decreased FCR but the FCR in these groups were still higher than that of NP fed birds from d 7 to 35 ($P < 0.001$). Livability was not affected during any experimental periods by either dietary treatment or temperature conditions ($P > 0.05$, Tables 3 and 4).

3.2. Carcass traits and organ weight

Results on relative carcass yield and organ weights collected on d 35 are presented in Table 5. No temperature × diet interactions were detected for carcass yield, fat pad weight, and weights of internal organs ($P > 0.05$); however, interactions were detected for absolute and relative ileum length ($P < 0.05$). In birds grown under NT, there was no difference in ileal length between the diets, but at cyclic WT, birds offered RP and RP-Arg had shorter ileum length than those offered NP, and ileum length was increased in birds fed the RP-Cit diet to the level of those fed the NP diet ($P < 0.05$) as shown in Table 5. Birds exposed to cyclic WT had decreased relative weights of the fat pad, liver, bursa, ileum, thigh and drumstick ($P < 0.05$), and there was a tendency for decreased relative breast meat yield ($P = 0.082$) but no differences in relative spleen weights ($P > 0.05$) compared to those exposed to NT (Table 5). Birds fed the RP diet had lower relative thigh and drumstick yields ($P < 0.05$), greater relative fat pad yield ($P < 0.05$), and tended to have lower relative breast meat yields ($P = 0.080$) compared to those fed the NP diet. Supplementation of Arg or Cit to the RP diet increased

Table 3
Growth performance of birds fed reduced protein diets supplemented with different arginine sources from d 7 to 21.

Treatment	FI, g	BWG, g	FCR	Livability, %
NP[1]	1,006	733	1.373[ab]	98.8
RP[2]	998	705	1.419[b]	98.8
RP-Arg[3]	968	706	1.359[a]	98.8
RP-Cit[4]	993	705	1.396[ab]	98.8
Pooled SEM	6.61	5.77	0.008	0.39
P-value	0.218	0.239	0.035	1.000

[a, b] Differing superscripts indicate significant differences between means ($P < 0.05$). As livability data were not normally distributed, they were tested for significance using the Kruskal–Wallis non-parametric test.
[1] Diet contained standard crude protein levels at 22.3% for grower phase (d 7 to 21).
[2] Diet contained reduced crude protein levels at 19.5% for grower phase (d 7 to 21).
[3] Reduced protein diet supplemented with 0.29% L-arginine for grower diets (d 7 to 21).
[4] Reduced protein diet supplemented with 0.29% L-citrulline for grower diets (d 7 to 21).

Table 4
Growth performance of birds fed reduced protein diets supplemented with different arginine sources from d 21 to 35 and the entire study (d 7 to 35).

Mean of main effects		d 21 to 35				d 7 to 35			
		FI, g	BWG, g	FCR	Livability, %	FI, g	BWG, g	FCR	Livability, %
Temperature	NT[1]	1,861[a]	1,174[a]	1.590	99.6	2,868[a]	1,885[a]	1.517	99.1
	WT[2]	1,728[b]	1,061[b]	1.636	99.3	2,707[b]	1,760[b]	1.542	97.6
Diet	NP[3]	1,837	1,213[b]	1.517[a]	100	2,843	1,934[a]	1.471[a]	98.8
	RP[4]	1,820	1,045[a]	1.743[c]	99.4	2,824	1,752[b]	1.609[c]	98.2
	RP-Arg[5]	1,777	1,109[ab]	1.605[b]	100	2,731	1,806[ab]	1.520[b]	98.8
	RP-Cit[6]	1,743	1,103[a]	1.587[b]	98.4	2,736	1,799[ab]	1.524[b]	97.6
Pooled SEM		18.17	16.54	0.01	0.31	23.72	20.57	0.01	0.49
P-value	Temperature	<0.001	<0.001	0.107	0.589	<0.001	0.001	0.166	0.148
	Diet	0.253	0.002	<0.001	0.267	0.209	0.009	<0.001	0.899
	Temperature × diet	0.917	0.691	0.433	–	0.956	0.902	0.695	–

[a, b, c] Differing superscripts indicate significant differences between means ($P < 0.05$). As livability data were not normally distributed, they were tested for significance using the Kruskal–Wallis non-parametric test and were not subjected to two-way ANOVA.
[1] Birds were raised at temperature following Ross 308 recommendation from d 0 to 21 and 24 °C from d 21 to 35.
[2] Birds were raised at temperature following Ross 308 recommendation from d 0 to 21 and 33 ± 1 °C for 6 h per day from d 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 for grower and finisher phases, respectively.
[6] Reduced protein diet supplemented with 0.29% and 0.28% L-citrulline for grower and finisher phases, respectively.

Table 5
Carcass yield and internal organ weights as percentages of body weight at d 35.

Treatment		Breast, %	Thigh and drumstick, %	Fat pad, %	Liver, %	Bursa of Fabricius, %	Spleen, %	Ileum weight, %	Ileum length, mm	Ileum length, mm/kg BW
NT[1]	NP[3]	13.40	23.28	1.02	2.48	0.27	0.11	1.09	83.2[bc]	0.0451[bc]
	RP[4]	11.96	20.72	1.20	2.51	0.25	0.10	1.03	76.9[ac]	0.0418[ac]
	RP-Arg[5]	13.13	22.94	1.12	2.59	0.22	0.11	1.03	77.6[ac]	0.0421[ac]
	RP-Cit[6]	13.17	21.68	1.10	2.43	0.24	0.12	1.04	77.8[ac]	0.0423[ac]
WT[2]	NP	12.74	22.31	0.87	2.25	0.20	0.11	1.05	85.6[c]	0.0471[c]
	RP	11.25	20.02	1.15	2.47	0.20	0.09	0.96	73.5[ab]	0.0405[ab]
	RP-Arg	11.31	20.29	0.96	2.17	0.20	0.11	0.86	71.5[a]	0.0393[a]
	RP-Cit	13.00	21.60	0.92	2.38	0.20	0.12	1.04	84.6[c]	0.0466[c]
Mean of main effects										
Temperature	NT	12.91	22.16[a]	1.11[a]	2.50[a]	0.24[a]	0.11	1.05[a]	78.9	0.0428
	WT	12.07	21.05[b]	0.98[b]	2.32[b]	0.20[b]	0.11	0.98[b]	78.8	0.0434
Diet	NP[3]	13.07	22.79[a]	0.94[a]	2.36	0.23	0.11[ab]	1.07	84.4[c]	0.0461[c]
	RP[4]	11.60	20.37[b]	1.18[b]	2.49	0.22	0.10[a]	0.99	75.2[ab]	0.0412[ab]
	RP-Arg[5]	12.22	21.62[ab]	1.04[ab]	2.38	0.21	0.11[ab]	0.95	74.5[a]	0.0407[a]
	RP-Cit[6]	13.08	21.64[ab]	1.01[ab]	2.41	0.22	0.12[b]	1.04	81.2[bc]	0.0444[bc]
Pooled SEM		0.24	0.26	0.03	0.05	0.006	0.003	0.019	0.99	0.0005
P-value	Temperature	0.082	0.036	0.005	0.039	<0.001	0.615	0.005	0.959	0.757
	Diet	0.080	0.011	0.011	0.776	0.515	0.045	0.165	<0.001	<0.001
	Temperature × diet	0.634	0.230	0.725	0.414	0.418	0.745	0.311	0.022	0.019

NT = thermoneutral temperature; WT = cyclic warm temperature; NP = normal protein; RP = reduced protein.
[a, b, c] Differing superscripts indicate significant differences between means ($P < 0.05$).
[1] Birds were raised at temperature following Ross 308 recommendation from d 0 to 21 and 24 °C from d 21 to 35.
[2] Birds were raised at temperature following Ross 308 recommendation from d 0 to 21 and 33 ± 1 °C for 6 h per day from d 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 for grower and finisher phases, respectively.
[6] Reduced protein diet supplemented with 0.29% and 0.28% L-citrulline for grower and finisher phases, respectively.

relative weights of breast and thigh and drumstick, and decreased relative fat pad weight to the levels of NP fed birds ($P > 0.05$). No differences in relative weights of liver, bursa of Fabricius, spleen, ileum, or ileum length were observed between NP and RP groups ($P > 0.05$). Supplementation of Cit but not Arg to RP increased relative spleen weight ($P < 0.05$).

3.3. Apparent ileal digestibility, serum uric acid, and gut permeability

No temperature × diet interactions were observed for apparent ileal digestibility, SUA, or serum FITC-d levels ($P > 0.05$) as shown in Table 6. The birds exposed to cyclic WT had higher ($P < 0.001$) serum FITC-d concentration on d 28 and higher ($P < 0.01$) SUA level on d 35 compared to those grown under NT conditions. Reduction of dietary CP resulted in increased IDNC ($P < 0.001$) but reduced IDE at d 21 ($P < 0.01$, Table 6). Ileal energy digestibility was not affected by any of the dietary treatments ($P > 0.05$, Table 6). The addition of Cit to the RP diet increased IDE to the level of the NP fed birds and increased IDNC to a level that was significantly higher than that of NP and RP-Arg fed birds ($P < 0.001$). Supplementation of Cit to the RP diet reduced the SUA level to a lower level than in birds fed the NP diet at d 21 ($P < 0.05$) whereas SUA levels of NP, RP and RP-Arg fed birds were not different ($P > 0.05$, Table 6). Similarly, the SUA level tended to be lower in birds fed the RP-Cit diet compared to those fed the NP diet at d 35 ($P = 0.065$).

Table 6
Serum uric acid and fluorescein isothiocyanate dextran (FITC-d) levels and apparent ileal energy and nitrogen digestibility.

Mean of main effects		FITC-d level d 28	Uric acid level d 21	Uric acid level d 35	IDE[1] as per DM d 21, kcal/kg	IDEC[2] d 21	IDNC[3] d 21
Temperature	NT[1]	0.066[a]	–	6.624[a]	–	–	–
	WT[2]	0.142[b]	–	7.400[b]	–	–	–
Diet	NP[3]	0.098	9.799[a]	7.705	3,321[b]	0.730	0.823[a]
	RP[4]	0.119	8.902[ab]	6.746	3,143[a]	0.711	0.842[bc]
	RP-Arg[5]	0.114	9.256[ab]	6.900	3,161[a]	0.712	0.835[ab]
	RP-Cit[6]	0.098	8.428[b]	6.697	3,239[ab]	0.731	0.847[c]
Pooled SEM		0.007	0.156	0.152	20.75	0.004	0.002
P-value	Temperature	<0.001	–	0.006	–	–	–
	Diet	0.194	0.014	0.065	0.006	0.184	<0.001
	Temperature × diet	0.387	–	0.408	–	–	–

IDE = ileal digestible energy; DM = dry matter; IEDC = ileal energy digestibility coefficient; INDC = ileal nitrogen digestibility coefficient; NT = thermoneutral temperature; WT = cyclic warm temperature; NP = normal protein; RP = reduced protein.
[a, b, c] Differing superscripts indicate significant differences between means ($P < 0.05$).
Statistical analysis on serum uric acid level at d 35 was performed on log-transformed data.
[1] Birds were raised at temperature following Ross 308 recommendation from d 0 to 21 and 24 °C from d 21 to 35.
[2] Birds were raised at temperature following Ross 308 recommendation from d 0 to 21 and 33 ± 1 °C for 6 h per day from d 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 for grower and finisher phases, respectively.
[6] Reduced protein diet supplemented with 0.29% and 0.28% L-citrulline for grower and finisher phases, respectively.

3.4. Bone morphology and quality

Results on bone parameters are presented in Tables 7–9. Interactions between dietary treatments and temperature were found for d 28 femur ash ($P < 0.05$). The cyclic WT increased femur ash in NP, RP, and RP-Cit fed birds. When birds were kept at NT, higher femur ash was observed in birds receiving RP-Arg and RP-Cit diets compared to that of the NP fed birds. Greater femur ash was observed in the NP fed birds compared to other treatments in birds raised under cyclic WT conditions at d 28 (Table 8). When birds were exposed to cyclic WT, no difference in femur ash between treatment groups was observed ($P > 0.05$) as shown in Table 8. The cyclic WT had no effect on absolute and relative femur weight, femur length, diameter, breaking strength, and Seedor index in the first week after the temperatures were increased on d 28. A greater absolute femur weight, Seedor index, length, and diameter were observed in NT birds compared to cyclic WT birds on d 35 ($P < 0.05$) as shown in Table 9. The cyclic WT had no effect on femur breaking strength observed at both d 28 and 35 ($P > 0.05$).

Birds fed the Arg-deficient RP diet had greater relative femur weight than birds fed the NP diet on d 21 ($P < 0.01$). Supplementation of Arg and Cit to the RP diet did not affect the examined femur parameters on d 21 ($P > 0.05$, Table 7). On d 28, Arg supplementation to the RP resulted in significant decreases in relative femur weight compared to RP treatment ($P < 0.01$, Table 8). On d 35, RP fed birds had lower femur ash ($P < 0.001$) and lower femur breaking strength compared to those fed the NP diet ($P < 0.01$). Both Arg and Cit addition to the RP diet increased femur ash and femur breaking strength to the levels that were not different to those of the NP fed birds at d 35 ($P > 0.05$; Table 9).

Table 7
Femur weight, ash, length, diameter and breaking strength at d 21.

Variable	NP[1]	RP[2]	RP-Arg[3]	RP-Cit[4]	SEM	*P*-value
Absolute weight[5], g	3.66	3.52	3.56	3.60	0.04	0.717
Relative weight[6], %	0.36[a]	0.39[b]	0.38[ab]	0.38[b]	0.00	0.003
Ash[7], %	47.5	47.2	46.9	46.7	0.18	0.452
Length[8], mm	51.1	50.7	50.6	50.9	0.17	0.729
Seedor index[9], mg/mm	31.4	30.8	31.4	31.5	0.26	0.770
Diameter, mm	6.88	6.85	6.92	6.77	0.05	0.704
Breaking strength, N	201	196	200	203	3.53	0.905

NP = normal protein; RP = reduced protein.
[a, b] Differing superscripts indicate significant differences between means ($P < 0.05$).
[1] Diet contained standard crude protein levels at 22.3% for grower phase (d 7 to 21).
[2] Diet contained reduced crude protein levels at 19.5% for grower phase (d 7 to 21).
[3] Reduced protein diet supplemented with 0.29% L-arginine for grower phase (d 7 to 21).
[4] Reduced protein diet supplemented with 0.29% L-citrulline for grower phase (d 7 to 21).
[5] Absolute femur weight was measured on the wet femur.
[6] Relative femur weight was calculated as a percentage of body weight.
[7] Femur ash was expressed as a percentage of oven-dry bone (%).
[8] Femur length, diameter, and breaking strength were measured on air dry bones.
[9] Bone Seedor index: the weight of oven-dry bone/bone length (mg/mm).

4. Discussion

4.1. Growth performance

Feeding diets deficient in Arg has been reported to decrease FI and BWG as compared to birds fed diets with sufficient levels of Arg (Castro et al., 2018). In the current study, BWG was only reduced in RP fed birds compared to birds fed the NP diet during the finisher phase (d 21 to 35) whereas FI was not affected by any of the dietary treatments. Chamruspollert et al. (2004) indicated that BWG in birds was mainly influenced by dietary FI ($R^2 = 0.90$). The similar BWG between birds fed the Arg-deficient RP diet and other groups during the grower phase in the current study might be attributed to the similar FI between these groups. The CP level of the grower RP diet used in the current study was 2.8% lower than the NP diet (19.5% vs. 22.3%). The addition of Arg or Cit to the RP diet decreased FCR to the levels that were not different to the NP treatment in the grower phase in the current study. Similar findings were obtained by previous investigators (Han et al., 1992; Corzo and Kidd, 2003a; Castro et al., 2018). The results of the current study also showed that supplementation of Arg or Cit to the RP diets reduced FCR but the FCR in these groups were still higher than birds fed the NP diet during the finisher phase. As Arg is a component of protein, and an essential AA for birds, inadequate provision of dietary Arg can directly influence protein synthesis at the level of translation (Kwak et al., 1999). Besides, the lower availability of Arg in the RP diet may cause an Arg:Lys imbalance and thus further reduce growth

Table 8
Femur weight, ash, length, diameter, and breaking strength at d 28.

Treatment		Absolute weight[7], g	Relative weight[8], %	Ash[9], %	Length[10], mm	Seedor index[11], mg/mm	Diameter, mm	Breaking strength, N
NT[1]	NP[3]	5.73	0.39	42.8^{ab}	59.3	45.5	8.21	271
	RP[4]	5.66	0.40	41.6^{a}	60.2	46.0	8.23	272
	RP-Arg[5]	5.41	0.37	43.8^{ab}	59.1	43.6	7.98	272
	RP-Cit[6]	5.35	0.38	43.1^{ab}	59.7	42.2	7.86	252
WT[2]	NP	5.48	0.38	44.6^{b}	58.5	44.7	8.22	299
	RP	5.24	0.40	43.0^{ab}	58.7	43.6	7.97	253
	RP-Arg	5.40	0.37	42.5^{ab}	59.0	44.2	7.86	256
	RP-Cit	5.29	0.38	43.4^{ab}	59.2	43.0	7.86	289
Mean of main effects								
Temperature	NT	5.54	0.39	42.8	59.6	44.3	8.07	267
	WT	5.35	0.38	43.4	58.9	43.9	7.98	274
Diet	NP	5.61	0.39^{ab}	43.7	58.9	45.1	8.22	285
	RP	5.45	0.40^{b}	42.3	59.4	44.8	8.10	262
	RP-Arg	5.40	0.37^{a}	43.1	59.1	43.9	7.92	264
	RP-Cit	5.32	0.38^{ab}	43.3	59.5	42.6	7.86	270
Pooled SEM		0.07	0.003	0.21	0.19	0.40	0.06	5.98
P-value	Temperature	0.150	0.468	0.180	0.174	0.584	0.444	0.555
	Diet	0.473	0.003	0.104	0.606	0.121	0.136	0.545
	Temperature × diet	0.674	0.697	0.023	0.548	0.446	0.851	0.234

NT = thermoneutral temperature; WT = cyclic warm temperature; NP = normal protein; RP = reduced protein.
[a, b] Differing superscripts indicate significant differences between means ($P < 0.05$).
[1] Birds were raised at temperature following Ross 308 recommendation from d 0 to 21 and 24 °C from d 21 to 35.
[2] Birds were raised at temperature following Ross 308 recommendation from d 0 to 21 and 33 ± 1 °C for 6 h per day from d 21 to 35.
[3] Diet contained standard crude protein levels at 20.9% for finisher phase.
[4] Diet contained reduced crude protein levels at 18.4% for finisher phase.
[5] Reduced protein diet supplemented with 0.29% and 0.28% L-arginine for grower and finisher phases, respectively.
[6] Reduced protein diet supplemented with 0.29% and 0.28% L-citrulline for grower and finisher phases, respectively.
[7] Absolute femur weight was measured on the wet femur.
[8] Relative femur weight was calculated as a percentage of body weight.
[9] Femur ash was expressed as a percentage of oven-dry bone (%).
[10] Femur length, diameter, and breaking strength were measured on air dry bones.
[11] Bone Seedor index: the weight of oven-dry bone/bone length (mg/mm).

Table 9
Femur weight, ash, length, diameter, and breaking strength at d 35.

Mean of main effects		Absolute weight[7], g	Relative weight[8], %	Ash[9], %	Length[10], mm	Seedor index[11], mg/mm	Diameter, mm	Breaking strength, N
Temperature	NT[1]	8.29^{a}	0.39	40.7	68.3^{a}	61.1^{a}	9.37^{a}	315
	WT[2]	7.77^{b}	0.40	40.5	67.3^{b}	58.0^{b}	9.07^{b}	311
Diet	NP[3]	8.35	0.39	41.9^{b}	67.7	60.9	9.30	352^{a}
	RP[4]	7.80	0.41	39.2^{a}	68.0	59.6	9.11	280^{b}
	RP-Arg[5]	7.99	0.40	40.6^{b}	67.6	59.4	9.26	316^{ab}
	RP-Cit[6]	7.97	0.39	40.7^{b}	67.9	58.3	9.21	305^{ab}
Pooled SEM		0.09	0.00	0.22	0.20	0.06	0.07	7.98
P-value	Temperature	0.004	0.413	0.691	0.013	0.005	0.019	0.798
	Diet	0.201	0.337	<0.001	0.935	0.447	0.745	0.009
	Temperature × diet	0.275	0.757	0.561	0.201	0.225	0.892	0.857

NT = thermoneutral temperature; WT = cyclic warm temperature; NP = normal protein; RP = reduced protein.
[a, b] Differing superscripts indicate significant differences between means ($P < 0.05$).
[1] Birds were raised at temperature following Ross 308 recommendation from d 0 to 21 and 24 °C from d 21 to 35.
[2] Birds were raised at temperature following Ross 308 recommendation from d 0 to 21 and 33 ± 1 °C for 6 h per day from d 21 to 35.
[3] Diet contained standard crude protein levels at 20.9% for finisher phase.
[4] Diet contained reduced crude protein levels at 18.4% for finisher phase.
[5] Reduced protein diet supplemented with 0.29% and 0.28% L-arginine for grower and finisher phases, respectively.
[6] Reduced protein diet supplemented with 0.29% and 0.28% L-citrulline for grower and finisher phases, respectively.
[7] Absolute femur weight was measured on the wet femur.
[8] Relative femur weight was calculated as a percentage of body weight.
[9] Femur ash was expressed as a percentage of oven-dry bone (%).
[10] Femur length, diameter, and breaking strength were measured on air dry bones.
[11] Bone Seedor index: the weight of oven-dry bone/bone length (mg/mm).

(Balnave and Brake, 2002). According to AMINOChick 2.0 software (Evonik Animal Nutrition), the optimal Arg-to-Lys ratio from d 7 to 21 and d 21 to 35 for broiler chickens are 1.04 and 1.05, respectively. The Arg-to-Lys ratio of the RP diet in the current study (calculated based on analyzed dietary AA levels) was lower than the NP diet (0.82 vs. 1.00 and 0.85 vs. 1.02 in the grower and finisher phase, respectively). Thus, lower growth performance in the RP group was expected and was hypothesized to be exacerbated under cyclic WT conditions. Supplementation of Arg or Cit was expected to increase Arg availability, correct any Arg:Lys imbalance, and increase growth relative to birds fed the RP diet. The results of the current study suggest that a moderate decrease in dietary CP level with adequate supplementation of crystalline AA or their metabolites (Cit in this case) in grower diets does not affect growth rate.

Reduced FI and BWG are common observations in birds grown in warm temperatures (Lisanne et al., 2016; Zhang et al., 2017; Xu

et al., 2018). However, heat stress may not affect nutrient digestibility in birds (Geraert et al., 1992; Faria Filho et al., 2007). Digestibility of dry matter, CP, crude fat, and nitrogen-corrected apparent metabolizable energy (AMEn) values have been reported to be unchanged in birds housed at 32 °C (Faria Filho et al., 2007). Likewise, Geraert et al. (1992) found that ME digestibility was not altered in heat-exposed broilers. Thus, although lower FI and BWG were observed in cyclic WT birds in the current study, their capacity to digest nutrients was not affected, resulting in similar FCR as compared to those housed under NT conditions.

Literature evidence has indicated that protein metabolism generates more heat than fat and carbohydrate metabolism in birds (Musharaf and Latshaw, 1999; Wu et al., 2019). Thus, the reduction of dietary CP during the heat stress period has been recommended by some to reduce heat production (Waldroup et al., 1976; Teeter, 1996). However, Macleod (1992) revealed that dietary protein did not influence heat production in birds housed at 32 °C. Chamruspollert et al. (2004) reported that interactions between Arg levels and temperature conditions on growth performance in broilers were only obtained when higher dietary Arg inclusion levels (0.152% and 0.252% vs. 0.152%, 0.252% and 0.352%) and higher temperature were applied (normal control: 22 °C vs. 25 °C; heat-treated: 32 °C vs. 35 °C). In which, increasing supplemental Arg level from 0.152% to 0.352% reduced BWG in birds reared at 25 °C; however, this effect was not observed in birds housed at 35 °C (Chamruspollert et al., 2004). In the current study, the low inclusion of dietary Arg used and the moderate difference between temperatures of NT and WT might be reasons for the lack of interaction between room temperature and dietary treatments observed.

4.2. Carcass traits and organ weight

Feeding low CP diets decreases muscle protein gain and increases abdominal fat in birds housed under normal (Belloir et al., 2017; Hilliar et al., 2019) or warm temperatures (Gonzalez-Esquerra and Leeson, 2005; Awad et al., 2015). Similar results were found in the current study. Fisher (1984) stressed that birds fed diets with higher ME-to-CP ratio tends to accumulate more abdominal fat than birds fed lower ME-to-CP ratio. Reducing the dietary CP level typically increases the ME-to-CP ratio and this may result in an energy surplus over that needed for protein deposition. This in turn leads to increased lipogenesis and abdominal fat pad accumulation (Awad et al., 2015). Increased breast meat yield and reduced abdominal fat in birds reared under normal (Al-Daraji et al., 2011; Wang et al., 2013; Castro et al., 2018) and heat stress conditions (Costa et al., 2001; Esser et al., 2017) have been reported with Arg supplementation. As a metabolite of Arg, Cit showed equal effects as Arg in improving carcass yield and reducing fat pad in the current study. Furthermore, the temperature × diet interaction in the current study showed ileal length at d 35 to be greater in birds fed RP-Cit (not RP-Arg) diet compared to RP fed birds only exposed to cyclic WT suggesting differential and more positive effects of Cit than Arg during warm temperatures.

Reductions in breast meat and increased fat deposition during heat exposure have been previously reported in broilers (Akit et al., 2005; Lu et al., 2007, 2019) and turkeys (Veldkamp et al., 2000). This effect may be associated with variation in energy distribution between fat and protein deposition in birds subjected to heat stress (Belhadj Slimen et al., 2016). In the current study, the relative fat pad weight was lower for birds exposed to cyclic WT compared to those exposed to NT. Previous reports have indicated that protein synthesis is reduced to a greater extent than protein degradation during heat stress resulting in lower overall protein accretion in meat (Temim et al., 1998, 2000).

Results on liver weight in the current study were consistent with those found by previous investigators showing relative weights of liver were not influenced by dietary CP reduction (Ardekani and Chamani, 2012; Awad et al., 2014a, 2015). Also, the results of the current study on organ weights are in agreement with Deng et al. (2005) who observed no difference in relative weights of the thymus, spleen, and bursa in Leghorn chickens receiving diets supplemented with Arg from d 1 to 28. Meanwhile, Kwak et al. (1999) indicated that Arg could considerably influence the development of these organs in birds, with a more conspicuous effect on the spleen than the bursa of Fabricius. In the current study, increased relative spleen weight was observed when Cit (but not Arg) was supplemented to the RP diet. This result may reflect the more beneficial effect of Cit compared to Arg on the development of the spleen that is supported by previous findings that Cit supplementation is more effective than Arg in increasing blood Arg level and therefore well matched with those found by Kwak et al. (1999). The liver is a primary source of lipolytic enzymes (Sobotka and Glick, 1934); hence, the decrease in liver weight observed in cyclic WT birds compared to NT birds in the current study might be associated with a reduction of lipolytic enzyme activity and hepatic lipogenesis (Balnave, 1972; Wagner et al., 1978; Belhadj Slimen et al., 2016). Importantly, the findings of the current study suggest that weights of liver, bursa of Fabricius, and ileum are more sensitive to changes in temperature conditions whereas spleen weight is more sensitive to the balance of dietary nutrients (CP level and sufficiency of Arg in this case). It has been speculated that the decrease in relative weights of the thymus, bursa, and liver in heat-exposed birds might due to the reduced FI resulting in less nutrients available for the proper development of these organs in the respective group (Bartlett and Smith, 2003). A similar situation might occur in the current study as lower FI was also observed in cyclic WT birds compared to the NT group.

4.3. Apparent ileal digestibility, serum uric acid, and gut permeability

Increased nitrogen digestibility in low CP diets as compared with normal CP diets has been reported in the literature (Han et al., 1990; Chung and Baker, 1991; Hilliar et al., 2019). A similar result was obtained in the current study. This was likely a result of highly digestible crystalline AA provided in the RP diet versus higher rates of protein-bound AA in the form of intact protein in the NP diet. Dietary supplementation of Arg has been reported to increase gut morphology and nutrient absorption in the small intestine and was speculated to be related to Arg roles in polyamines production, cell mitosis, and/or nitric oxide activation (Khajali et al., 2014; Abdulkarimi et al., 2019). Polyamines such as putrescine, spermine, and spermidine are known as biogenic amines playing a crucial role in the development of the small intestine, colonic mucosa, and cell growth (Löser et al., 1999). In the current study, the lack of Arg effect on ileal nutrient digestibility might be a result of a relatively lower dose as compared with other studies. Interestingly, Cit increased ileal energy and nitrogen digestibility to a greater extent than Arg in the current study. In both mammals and birds, ornithine is mainly used for the synthesis of Cit, polyamines, and glutamate (Wiesinger, 2001). As ornithine is the direct precursor of polyamines, dietary Cit supplementation might increase ornithine availability for polyamines production with subsequent effects on improving gut morphology and nutrient digestibility. In addition, it is worth noting that birds are unable to synthesize ornithine from Arg because they lack the key enzymes involving in this process (Khajali and Wideman, 2010). Thus, unlike Cit, Arg can only indirectly influence the polyamine synthesis through Arg-Cit conversion. This fact might partly explain the more beneficial effects of Cit

compared to Arg supplementation on nutrient digestibility in the current study.

Increasing dietary CP levels have been reported to increase SUA levels in birds (Hernández et al., 2012; Hilliar et al., 2019). The findings of the current study were consistent with those previously reported. The higher SUA levels observed in high CP fed birds may be associated with excessive intake of AA and the lower SUA level may reflect reduced AA ingestion particularly for glycine because this AA is necessary for the synthesis of uric acid molecules (Namroud et al., 2008; Ospina-Rojas et al., 2013). With less excessive AA in the diet, birds used dietary nitrogen more efficiently and produce less uric acid. This was consistent with the ileal nitrogen digestibility results in the current study. As SUA is a product of dietary excessive or imbalanced AA (Namroud et al., 2008), the reduction in SUA level in RP-Cit fed birds in the current study might indicate more efficiency in Arg utilization for its metabolic pathways in the RP-Cit group. Previous studies have indicated that ingested Arg is largely degraded to nitrogenous wastes such as uric acid at its first-pass metabolism from the liver, where arginase enzyme activity is highly active (Allerton et al., 2018). Citrulline, otherwise, is absorbed in the kidney and converted to Arg through successive actions of argininosuccinate synthetase and argininosuccinate lyase before it is released into the blood stream as Arg (El-Hattab et al., 2012). Thus, the difference in metabolic pathways of Arg and Cit may partly explain their differential effects on the SUA level observed in the current study.

The intestinal mucosa is important for gut barrier integrity (Turner, 2009; Steed et al., 2010). Gut barrier integrity was reduced by cyclic WT in the current study as shown by higher FITC-d levels in blood serum on d 28 compared to the counterparts grown under NT conditions. Similar results have been reported by others (Varasteh et al., 2015; Zhang et al., 2017; Wu et al., 2018). Also, heat stress has been reported to increase the uric acid level in the blood (Kataria et al., 2008). Similar findings were observed in the current study and likely due to reduced nitrogen digestibility with more nitrogen degraded to uric acid as a result of cyclic WT conditions.

4.4. Bone morphology and quality

Dietary protein has a significant effect on bone quality because protein is an integral part of the bone matrix and affects calcium absorption and excretion (Heaney and Layman, 2008). Lower femur width, humerus weight, and humerus Seedor index in birds fed reduced protein diets compared to those fed normal protein diets was reported by Bruno et al. (2007). In the current study, birds receiving Arg-deficient RP diets had lower femur ash and lower femur breaking strength than birds fed the NP diet at d 35. Dietary Arg plays an important role in bone development by influencing the process of mineral metabolism, bone mineralization, and the formation of collagen and connective tissues (Corzo et al., 2003b; Silva et al., 2012; Castro et al., 2018). Castro et al. (2018) observed no difference in bone growth rate between birds fed diets with deficient (70% of Ross 308 recommended level) or sufficient levels of Arg. However, significantly lower bone mineral density was found in birds fed Arg-deficient diets compared to those fed the Arg-sufficient diets at d 42 (Castro et al., 2018). The femur ash results of the current study further supported the positive effects of Arg supplementation on bone mineralization documented in the literature. The results of the current study showed that Arg and Cit were both effective in improving bone morphology and quality in reduced CP diets under NT conditions.

Decreased bone morphology and ash content in heat stress birds have been documented in the literature (Bruno et al., 2000; Vakili et al., 2010; Mosleh et al., 2018). Sgavioli et al. (2016) found negative effects of heat stress on femur weight, diameter, and mineral contents in broilers. The finding of the current study was in agreement with those previously reported. Heat stress has been also reported to reduce bone strength and cause considerable skeletal problems in fast-growing broilers (Post et al., 2003; Abioja et al., 2012). The milder temperatures applied in the current study compared to other studies may be the reason for the lack of cyclic WT effects on the bone breaking strength. In the current study, both Arg and Cit supplementation to the RP diet resulted in higher femur ash (d 28) at NT rather than at cyclic WT whereas femur ash (d 28) of birds fed the NP diet was greater in cyclic WT than NT. As levels of Arg/Arg-equivalent in RP-Arg, RP-Cit and NP treatments in the current study were similar, this result might be attributed to the lower levels of other components in the RP-Arg and RP-Cit diets compared to the NP diet such as dietary electrolyte balance, glycine, phenylalanine, leucine, histidine, or other non-essential AA. These dietary factors have been reported to play important roles in birds growing under warm conditions (Ahmad and Sarwar, 2006; Waldroup, 2007; Awad et al., 2014b, 2015). Further work is warranted to clarify this speculation.

5. Conclusions

The results of the current study showed that birds fed the RP diet supplemented with either Arg or Cit had similar FI, BWG, FCR, bone morphology, and bone strength compared to NP fed birds during the grower phase (d 7 to 21). Supplementation of Cit to the RP diet resulted in greater ileal nitrogen digestibility compared to the RP-Arg fed birds and lower SUA level compared to the NP fed birds on d 21 indicating its potential as an effective alternative source of Arg in broiler diets. During the finisher phase (d 21 to 35) and overall period (d 7 to 35), birds fed the RP diet supplemented with either Arg or Cit; however, had higher FCR compared to those fed the NP diet regardless of temperature conditions. Thus, reduction of dietary protein level by 2.5 percentage points with adequate supplementation of crystalline AA or its metabolites (Cit in this case) could maintain growth performance during the grower phase, but this strategy might reduce bird performance during the finisher phase. Diet and temperature interactions were not observed for performance measurements, but they were significant for ileal length (d 35) and femur ash (d 28) indicating that RP-Cit and NP diets gave more positive effects on these parameters than RP-Arg diet during warm temperatures. This result suggests that feeding RP-Cit or NP diet might help to increase nutrient digestibility and bone mineralization under warm temperatures. Further work is warranted to determine optimal levels of Arg and/or Cit to support growth performance in birds fed various protein levels under different heat stress scenarios (chronic or acute). In addition, interactions between Arg, Lys, and dietary electrolyte balance during heat stress should be investigated.

Author contributions

Hiep Thi Dao - Conceptualization, Methodology, Formal analysis, Validation, Writing original, Review, Statistics and editing; **Nishchal K. Sharma** - Review, editing and validation; **Emma Bradbury** - Review and editing. **Robert A. Swick** - Conceptualization, Review and editing, Supervision, Project administration, Resources.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal

interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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