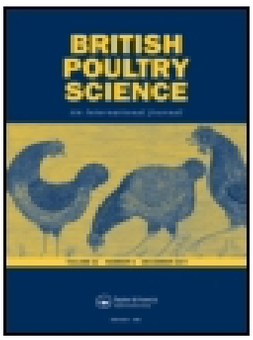

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Interactive effects of temperature and dietary supplementation of arginine or guanidinoacetic acid on nutritional and physiological responses in male broiler chickens

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ABSTRACT

1. The aim of this experiment was to study the interactive effect of rearing temperature and dietary supplementation of arginine (Arg) or guanidinoacetic acid (GAA) on performance, gut morphology and ascites indices in broiler chickens raised under the same condition in the first 2 weeks and then reared under normal (23–26°C) or subnormal (17°C) ambient temperatures for the next 3 weeks.
2. This experiment was conducted as a split plot with 900 Ross 308 male broiler chicks that were allocated to two houses (as main plots); each consisted of 5 treatments (as sub-plots) with 6 replicates of 15 birds. The 5 diets were (1) control, (2) control + 0.60 g/kg GAA, (3) control + 1.20 g/kg GAA, (4) control + 0.86 g/kg Arg and (5) control + 1.72 g/kg Arg.
3. Feed intake (0–35 d) of birds fed on a diet containing 1.2 g GAA/kg and reared under normal temperature was reduced compared to control fed birds. Birds fed on a diet containing 1.72 g/kg Arg and reared under subnormal temperature had higher weight gain compared to those fed on control or GAA-added diets in overall study period.
4. Supplementation of diets with Arg alleviated the adverse effect of cold stress as reflected by reduction in blood haematocrit (41% vs. 37%), and right ventricle to total ventricle ratio (0.28 vs. 0.25) at 35 d of age. Addition of Arg to the diet of birds reared under cold stress resulted in a higher jejunal villus surface area compared to those fed on control or GAA-added diets.
5. Findings of this study revealed that Arg or GAA supplementation of diets did not affect performance of birds under normal temperatures, but Arg supplementation of the diet significantly alleviated the adverse effect of cold stress on performance, gut development and ascites syndrome. In addition, GAA supplementation at 1.2 g/kg improved jejunal villus surface area in birds raised under subnormal temperature.

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Ascites; performance; arginine; guanidinoacetic acid; blood indices; gut health

Introduction

Ascites syndrome in poultry is a cardiovascular metabolic disorder characterised by accumulation of ascitic fluid in the abdominal cavity and around the heart (Cahaner, 2011). High growth rate in modern broilers has increased the metabolic demand for oxygen due to higher metabolic rate (Baghbanzadeh and Decuypere, 2008; Kalmar *et al.*, 2013) and also resulted in reduced relative heart and lung size, a result of limits on cardiopulmonary capacity (Kalmar *et al.*, 2013). Therefore, the modern broilers, especially males, are more susceptible to the development of ascites (Baghbanzadeh and Decuypere, 2008). Moreover, cold stress increases oxygen demand and those broilers that fail to fully supply oxygen may develop ascites (Cahaner, 2011).

Nain *et al.* (2009) estimated worldwide industry losses due to ascites to be one billion dollars a year. Losses are due to reduced overall flock feed efficiency, increased mortality of the heaviest birds, higher mortality during transport to the processing plant and higher condemnation of carcasses during processing (Hasanpur *et al.*, 2015).

Arginine (Arg) is the fifth-most limiting amino acid in maize–soybean meal diets for broiler chickens (Waguespack *et al.*, 2009). Birds lack a urea cycle so feed quantities of Arg must be sufficient to support protein synthesis, growth, feathering and other key biological functions (Khajali and Wideman, 2010). Supplementation of Arg to balanced rations (formulated based on NRC, 1994 recommendations) has been reported to have beneficial effects, including lymphoid organ development, on broilers raised under normal environment (Kwak *et al.*, 1999; Jahanian, 2009; Ruiz-Feria and Abdukalykova, 2009). Besides, Arg supplementation (10 g/kg) could improve gut morphology (a characteristic of gut health) and thus might affect the metabolism of this high oxygen demanding organ (Yen *et al.*, 1989; Laudadio *et al.*, 2012; Khajali *et al.*, 2014). Conditions like cold stress with increases in oxygen demand might increase the Arg requirement of birds. Providing extra Arg could reduce mortality due to ascites (Wideman *et al.*, 1995; Tan *et al.*, 2007) and improve performance of broiler chickens raised under cold temperature conditions (Saki *et al.*, 2013). Other studies revealed that supplementation of Arg to the diets formulated based on the NRC (1994) did not affect overall performance (Ruiz-Feria *et al.*, 2001; Saki *et al.*, 2013) or mortality (Ruiz-Feria *et al.*, 2001) of broilers reared under cold temperature. Extra Arg added to the diet could reduce ascites in broilers by elevating plasma nitric oxide levels (Saki *et al.*, 2013). Nitric oxide, produced from Arg, is a potent vasodilator that directly relaxes vascular smooth muscle and modulates or inhibits the production and release of vasoconstrictors such as serotonin and endothelin-1 (Wideman *et al.*, 2013). Also, Arg supplementation

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would decrease haematocrit in birds (Khajali *et al.*, 2014) and as a result reduces the workload on the heart. Reports also show that in-ovo injection of Arg could reduce heterophil (H) to lymphocyte (L) ratio (H/L), an indicator of sustained stress, in birds raised under cold temperature (Khajali *et al.*, 2008; Saki *et al.*, 2013).

Guanidinoacetic acid (GAA) is synthesised in the liver and kidney from Arg and glycine (Abudabos *et al.*, 2014) and is a natural precursor of creatine in the vertebrate body (Michiels *et al.*, 2012). The need for GAA is higher in broilers fed on all vegetable diets which lack creatine, and in young fast-growing chicks with a high need for creatine to support muscle growth (Michiels *et al.*, 2012). The amount of GAA synthesised in these situations might be insufficient to provide the requirement of fast-growing birds (Abudabos *et al.*, 2014). Results of two experiments demonstrated that GAA supplementation at the rate of 0.6 or 1.2 g/kg led to better weight gain (WG) and feed conversion (FCR) of birds (Ringel *et al.*, 2008; Michiels *et al.*, 2012).

Additional dietary Arg might reduce ascites incidence in broilers (Saki *et al.*, 2013), and Baker (2009) had hypothesised that dietary GAA could spare dietary Arg, in the same manner as dietary creatine. To support this, Dilger *et al.* (2013) showed that in practical vegetable diets (containing 1% Arg, and thus deficient), GAA could be an efficacious replacement for dietary Arg for young birds. Accordingly, it was hypothesised that administration of GAA in the diet, as an Arg-sparing compound, may improve performance and reduce mortality due to ascites in broiler chickens. Therefore, this study was conducted to compare the effect of dietary supplementation of Arg and GAA on performance, blood parameters and gut morphology of male broilers reared under normal or suboptimal temperatures.

Materials and methods

Experimental design, diets and environmental conditions

This experiment was conducted in two poultry houses (1080 m above sea level) that were identical regarding dimensions, and all the equipment, except that one of the houses were equipped with air-conditioning systems in order to be able to reduce the temperature. In each house, there were 30 pens, which were blocked (6 block/house) to account probable differences in housing temperature. Then, diets were randomised to pens within block (5 diets/block), and 15 birds were allocated to each pen (in total 450 birds/temperature regime). Birds were reared in 2 m^2 floor pens on 6 cm of wood shavings with *ad libitum* access to feed and water; provided through a tube feeder and nipple waterers. Chicks received a commercial maize–soybean meal diet formulated according to 2014 Ross 308 nutrient recommendations (Table 1) in mash form with the following supplementations: (I) control, no supplement; (II) 0.6 g/kg GAA (CreAMINO®, Evonik Degussa Gmbh, Essen, Germany); (III) 1.2 g/kg GAA; (IV) 0.86 g/kg Arg (L-arginine, KYOWA HAKKO BIO CO. LTD, Tokyo, Japan); (V) 1.72 g/kg Arg. The GAA supplementation was based on the Evonik recommendations (0.6 and 1.2 g/kg). Here, purity of CreAMINO® is 96%, and molecular weight of GAA is 117.11 g/mol, which accounts for 4.92 and 9.82 mol of GAA in every 0.6 or 1.2 g/kg CreAMINO®, respectively. Arginine supplemented as a pure source (100%) at isomolar levels relative to GAA by multiplying 4.92 and 9.82 mol into its molecular weight (174.2 g/mol). Arginine and GAA were added to the basal diet in substitution for an equal weight of maize. The lighting schedule was 23 h light and 1 h dark from d 1 to the end of the experiment at 35 d of age. The temperature of both houses were set at 32°C for the first week and then reduced to 29°C in the second week. Then, temperature of one house was gradually decreased by approximately 3°C/week from d 14 until it reached 23°C

Table 1. Composition of basal diets (as fed basis, g/kg)[a]

	Starter diet (0–10 d)	Grower diet (11–24 d)	Finisher diet (25–35 d)
Ingredient (%)			
Maize (74.1 g/kg crude protein)	526.6	554.7	610.8
Soybean meal (446.9 g/kg crude protein)	380	355.4	300
Soybean oil (37.71 MJ/kg)	41	44	48
Dicalcium phosphate	23	20.1	15
Calcium carbonate (370 g/kg Calcium)	10.8	10	10.8
Sodium chloride	2.5	2.6	2.6
Sodium bicarbonate	1.9	1.6	1.6
DL-Methionine (990 g/kg)[b]	3.9	3.2	2.9
L-Lysine hydrochloride (780 g/kg)[c]	3.4	2.2	2.2
L-Threonine (985 g/kg)[d]	1.9	1.2	1.1
Vitamin premix[e]	2.5	2.5	2.5
Trace mineral premix[f]	2.5	2.5	2.5
Calculated nutrient[g]			
Metabolisable energy (MJ/kg)	12.77	13.00	13.40
Crude protein	215.6	204.7	183.8
Dry matter	887.2	885.4	883.0
Ether extract	61.4	64.9	70.0
Total phosphorus	8.0	7.4	6.3
Available phosphorus	4.8	4.38	3.9
Calcium	9.7	8.7	7.9
Chlorine	2.5	2.3	2.3
Sodium	1.6	1.6	1.6
Potassium	9.2	8.7	7.8
Digestible methionine	6.7	5.9	5.4
Digestible methionine + cysteine	9.6	8.7	8.0
Digestible lysine	13.0	11.5	10.3
Digestible arginine	13.9	12.3	11.0
Digestible threonine	8.7	7.7	6.9
Linoleic acid	13.1	13.6	14.6
Dietary cation–anion balance (meq/kg)	234.6	227.5	204.4
Analysed nutrients (g/kg)			
Crude protein	216.0	205.4	183.2
Ether extract	62.1	66.3	70.5
Dry matter	885.0	888.3	886.4

[a]The supplemental guanidineacetic acid (0.6 and 1.2 g/kg) and arginine (0.86 and 1.72 g/kg) were replaced with similar amount of maize to provide the four experimental diets beside the control (basal) diet in every feeding period.
[b]MetAMINO, Evonik Degussa Gmbh, Essen, Germany.
[c]L-Lysine HCl, AJINOMOTO EUROLYSINE S.A.S, Paris, France.
[d]ThreAMINO, Evonik Degussa Gmbh, Essen, Germany.
[e]Supplied per kg diet: retinol 18 mg, cholecalciferol 4 mg, α-tocopherol acetate 36 mg, vitamin K3 2 mg, vitamin B_1 1.75 mg, vitamin B_2 6.6 mg, niacin 9.8 mg, pantothenic acid 29.65 mg, vitamin B_6 2.94 mg, folic acid 1 mg, vitamin B12 0.015 mg, biotin 0.1 mg, choline chloride 250 mg and ethoxyquin 1 mg.
[f]Supplied per kg diet: Mn 99.2 mg, Fe 50 mg, Zn 84.7 mg, Cu 10 mg, I 0.99 mg, Se 0.2 mg.
[g]Analysed by Evonik Industries AG animal nutrition analytical lab for crude protein (AMINOProx®), amino acids (AMINONIR®), ether extract (AMINOProx®), dry matter (AMINOLab®) and total and phytate phosphorous (AMINOProx®) contents. The amount of amino acids in the diets was calculated based on amino acid content of feed ingredients and the standardised ileal amino acid digestibility values reported by Lemme *et al.* (2004).

by d 28 of age and remained constant thereafter. The temperature in the second house was reduced to 17°C within 8 h on d 14 and kept constant until the end of the experiment to induce ascites in chickens. Temperature in each house was controlled with the use of a central heating system, and in case of second house air conditioners (after d 14). Temperature controlling equipment were in connection with thermostats and thermometers that were located at three different points per house, and temperature were recorded every 8 h. The experimental protocol was reviewed and approved by the Animal Care Committee at the Ferdowsi University of Mashhad.

Performance and ascites mortality

Chicks were weighed in groups of 15 immediately on arrival, and allocated to pens in each of the two houses. Similar mean body weight was obtained in all pens of both houses (43 ± 1.2 g). The pen weight of birds was recorded at the end of the study (d 35). Feed intake (FI) was also recorded on a per pen basis for the whole experimental period. WG and FI were calculated for all replicate pens for the overall study period (0–35 d), and mortality adjusted FCR were calculated based on Dersjant-Li *et al.* (2014).

Ascites mortalities were recorded daily as recognised by the accumulation of fluid in the abdominal cavity and pericardium (Varmaghany *et al.*, 2013) and RV to TV ratio (RV/TV) >0.25 (Saki *et al.*, 2013).

Blood and plasma parameters

At 35 d of age blood was collected via wing vein in 2-ml tubes containing EDTA from two birds per replicate pen representative of the average body weight of the pen. An aliquot of blood from each bird was immediately prepared for blood smears: spread on microscope slides, dried and fixed with methanol. Smears were stained with May-Grunwald and Giemsa stains (Lucas and Jamroz, 1961). For the differentiation of white blood cells count, the slides were observed under light microscope and 100 white blood cells were numerated in each sample. Percent of H, L and H/L ratio were calculated.

The remaining portion of blood was stored on dry ice for at most 1 h prior to analysis and used to detect haematocrit by centrifugation of capillary tubes containing blood samples at 11 500*g* for 10 min in a microhaematocrit centrifuge (Behdad Haematocrit, Labtron Co., Tehran, Iran). The plasma fraction from the capillary tube was immediately used to detect plasma total protein with the use of a refractometer (Model SPR-NE, Atago, Tokyo, Japan).

Internal organ assessment

The two birds per pen selected for blood samples (35 d of age) were sacrificed by decapitation, then carcass weight, as well as weight of heart, spleen and bursa were recorded.

For the RV/TV ratio determination, first the pericardium, peripheral adipose tissues and atria were removed from the heart (Varmaghany *et al.*, 2013). Then, the RV was dissected from the LV and septum. After this, the RV and TV were weighed and RV/TV was calculated.

Finally, 1-cm length from the mid-section of the jejunum were dissected and flushed with distilled water, then fixed in 10% formalin as previously described (Khodambashi Emami *et al.*, 2012), and cross sections were prepared by the use of a microtome (Rotary Microtome, Model MK1120, Pooyanmedical Co., Mashhad, Iran). There were three cross sections per sample (therefore, 36 cross sections for each treatment), and 10 measurements per cross section (for a total of 360 measurements per treatment). The formula for calculating villus surface area (VSA) was $2\pi \times (VW/2) \times VH$, where VH indicates villus height and VW stands for villus width that were measured at the middle point of the villus.

Statistical analysis

Data of this experiment were analysed as a split plot with temperature assigned to the whole plot and diet assigned to the sub plot. All data were tested for residual normality, and proved to be normal. MIXED procedure of SAS® software (SAS Institute, 2004) were used for data analysis and the significant difference between treatments means were determined by the Tukey test ($P \leq 0.05$).

Results

Performance and ascites mortality

The effect of dietary supplemental GAA and Arg on overall performance (0–35 d) in birds reared under normal or cold stress is shown in Table 2. The interaction effects of temperature and dietary treatment were pronounced ($P \leq 0.05$) for WG through the whole experimental period. Supplementation of Arg to diet of birds with cold stress improved their WG compared to other cold-stressed birds, whereas all the dietary treatments had similar WG under normal temperature. Also, birds fed on the 1.72 g/kg Arg supplemented diet with cold stress had statistically similar

Table 2. Effect of supplementing guanidinoacetic acid (GAA) and arginine (Arg) to the diet on overall (0–35 d) performance in broiler chickens grown in normal or cold temperatures.

Temperature	Diet supplementation (g/kg)	Weight gain (g/b)	Feed intake (g/b)	Feed conversion ratio
Normal	Control	2021^{a}	3461^{bcd}	1.71
	GAA (0.6)	1975^{ab}	3367de	1.70
	GAA (1.2)	1945^{abc}	3251^{f}	1.67
	Arg (0.86)	2004^{ab}	3427^{cde}	1.71
	Arg (1.72)	1986^{ab}	3359^{def}	1.68
Cold	Control	1827^{d}	3514^{bc}	1.92
	GAA (0.6)	1861^{cd}	3549^{ab}	1.90
	GAA (1.2)	1831^{d}	3468^{bcd}	1.89
	Arg (0.86)	1925^{bc}	3526^{bc}	1.83
	Arg (1.72)	1968^{ab}	3635^{a}	1.84
Temperature				
Normal		1986	3370	1.69^{b}
Cold		1883	3538	1.88^{a}
Diet supplementation (g/kg)				
Control		1921	3487	1.81
GAA (0.6)		1918	3458	1.80
GAA (1.2)		1886	3360	1.78
Arg (0.86)		1963	3477	1.77
Arg (1.72)		1974	3490	1.76
SEM		0.9	1.1	0.023
P-values				
Temperature		<0.001	<0.001	<0.001
Diet		<0.001	0.010	0.162
Temperature × diet		<0.001	0.036	0.223

$^{a–f}$Means in the same column with no common superscript letter differ significantly ($P < 0.05$).

WG compared to those fed on control diet and grown under normal temperature.

The interaction effect of temperature and dietary treatment was also significant for FI ($P \leq 0.05$) through the whole experimental period. Birds fed on the diet supplemented with 1.72 g/kg Arg under cold stress consumed more feed than those fed on GAA/Arg supplemented diet under normal temperature. Under normal ambient temperature, birds fed on the diet containing 1.2 g/kg GAA had lower FI ($P \leq 0.05$) compared to those fed on the control diet.

Feed-to-gain ratio was better ($P \leq 0.05$) in birds grown under normal temperature than those in the cold stress, but none of the dietary treatments had any influence on FCR in birds grown under either temperature regime.

Ascites indices

Total and ascites-related mortality is shown in Table 3. The main effect of temperature led to higher ($P \leq 0.05$) total mortality of birds in the cold temperature compared to normal temperature. However, dietary treatment had no effect on total mortality. In contrast, mortality due to ascites was influenced by temperature × diet interaction. Ascites-related mortality was lower ($P \leq 0.05$) in birds fed on a diet supplemented with 0.86 g/kg Arg and reared under cold temperature than those cold stressed fed on control or GAA supplemented diets, but not different ($P > 0.05$) than those grown under normal temperature.

Blood indices

The effect of temperature or dietary supplementation of GAA or Arg on plasma total protein, haematocrit and H/L is shown in Table 3. Cold-stressed birds had lower plasma total protein ($P \leq 0.05$) than those grown under normal temperature, but there was no effect of dietary treatment on plasma total protein. A temperature by diet interaction observed as supplementation of Arg at either rate of 0.86 and 1.72 g/kg reduced the haematocrit and H/L ($P \leq 0.05$) values compared to those fed on the control diet in the same cold temperature house; however, there were no effect of dietary treatments under normal temperature.

Internal organ assessment

RV to total ventricle ratio was decreased by supplementation of Arg to the diet under cold temperature ($P \leq 0.05$) compared to the control fed birds, and reached the level similar ($P > 0.05$) to control birds reared under normal temperature. RV/TV ratio >0.25 (Saki *et al.*, 2013) was considered as a sign of ascites. Therefore, only cold-stressed birds fed on a diet supplemented with 0.86 g/kg Arg were considered as normal (Table 3).

The effect of dietary supplementation of GAA/Arg on relative internal organ weights (relative to body weight) in broilers grown under normal or cold environmental temperature houses is shown in Table 4. The temperature × dietary treatment interaction was significant for relative weight of carcass and bursa. This resulted in an improved relative weight of carcass in cold-stressed birds fed on a diet supplemented with 0.86 g/kg Arg, and this was comparable to those fed on a diets supplemented with either Arg or GAA and grown in normal temperature with the exception of those fed on a diet supplemented with 1.72 g/kg Arg. Relative bursa weight was lower ($P \leq 0.05$) in birds fed on a diet supplemented with 1.72 g/kg Arg than other birds grown in the subnormal temperature house with the exception of those on diets supplemented with 0.86 g/kg Arg. The main effect of temperature led to higher ($P \leq 0.05$) relative spleen weight in birds reared under cold stress than those under normal temperature. In addition, birds receiving a diet supplemented with 1.72 g/kg Arg had lower ($P \leq 0.05$) relative spleen weight compared to other dietary treatments.

Table 3. Effect of supplementing guanidinoacetic acid (GAA) and arginine (Arg) to the diet on blood indices (at d 35), total and ascites related mortality (0–35 d), and right to total ventricle ratio (RV/TV) in broiler chickens grown in normal or cold temperatures.

		Parameters					
					Mortality (%)		
Temperature	Diet supplementation (g/kg)	Plasma total protein (g/dl)	Haematocrit (%)	H/L	Total	Ascites	RV/TV
Normal	Control	3.28	$35.8^{c,d}$	$0.48^{d,e}$	4.44	$1.11^{c,d}$	$0.24^{c,d}$
	GAA (0.6)	3.15	$36.2^{c,d}$	$0.49^{d,e}$	4.44	$2.22^{c,d}$	$0.23^{c,d}$
	GAA (1.2)	3.21	35.3^{d}	$0.51^{c,d,e}$	5.55	0.00^{d}	0.22^{d}
	Arg (0.86)	3.16	$36.8^{c,d}$	0.46^{e}	6.66	$1.11^{c,d}$	$0.25^{b,c,d}$
	Arg (1.72)	3.31	35.2^{d}	$0.51^{c,d,e}$	3.33	0.00^{d}	$0.24^{c,d}$
Cold	Control	2.98	41.0^{a}	0.60^{a}	10.00	$7.77^{a,b}$	$0.28^{a,b}$
	GAA (0.6)	3.15	$39.7^{a,b}$	$0.58^{a,b}$	8.88	$7.77^{a,b}$	0.29^{a}
	GAA (1.2)	3.00	40.3^{a}	$0.55^{a,bc}$	10.00	8.88^{a}	$0.28^{a,b}$
	Arg (0.86)	3.13	$37.2^{c,d}$	$0.55^{b,c}$	7.77	$3.33^{c,d}$	$0.25^{b,c,d}$
	Arg (1.72)	3.20	$37.8^{b,c}$	$0.52^{c,d}$	6.66	$4.44^{b,c}$	$0.26^{a,b,c}$
Temperature							
Normal		3.22^{a}	35.9	0.49	4.66^{b}	0.888	0.24
Cold		3.09^{b}	39.2	0.56	8.66^{a}	6.44	0.27
Diet supplementation (g/kg)							
Control		3.13	38.4	0.54	6.66	4.44	0.26
GAA (0.6)		3.15	37.9	0.53	6.66	4.99	0.26
GAA (1.2)		3.10	37.8	0.53	7.77	4.44	0.25
Arg (0.86)		3.15	37.0	0.50	7.21	2.22	0.25
Arg (1.72)		3.25	36.5	0.52	4.99	2.22	0.25
SEM		0.063	0.814	0.020	0.601	0.744	0.012
P-values							
Temperature		0.002	0.001	0.004	0.012	<0.001	0.001
Diet		0.199	0.139	0.395	0.836	0.466	0.640
Temperature × diet		0.135	0.028	0.047	0.850	0.040	0.019

$^{a-e}$Means in the same column with no common superscript letter differ significantly ($P < 0.05$).

Table 4. Effect of supplementing guanidinoacetic acid (GAA) and arginine (Arg) to the diet on relative internal organs weight (relative to live weight) in broiler chickens grown in normal or cold temperatures.

		Relative weight (%)		
Temperature	Diet supplementation (g/kg)	Carcass	Bursa	Spleen
Normal	Control	71.3^{ab}	0.128^{cd}	0.068
	GAA (0.6)	70.8^{ab}	0.118^{de}	0.075
	GAA (1.2)	71.6^{ab}	0.138^{abc}	0.068
	Arg (0.86)	70.6^{ab}	0.125^{cde}	0.071
	Arg (1.72)	71.9^{a}	0.113^{e}	0.059
Cold	Control	68.6^{c}	0.147^{ab}	0.110
	GAA (0.6)	68.6^{c}	0.145^{ab}	0.121
	GAA (1.2)	69.1^{c}	0.157^{a}	0.118
	Arg (0.86)	70.5^{b}	0.133^{bc}	0.107
	Arg (1.72)	69.8^{bc}	0.125^{cd}	0.085
Temperature				
Normal		71.3	0.125	0.069^{b}
Cold		69.3	0.140	0.108^{a}
Diet supplementation (g/kg)				
Control		70	0.137	0.089^{a}
GAA (0.6)		69.7	0.132	0.098^{a}
GAA (1.2)		70.3	0.145	0.093^{a}
Arg (0.86)		70.6	0.129	0.082^{a}
Arg (1.72)		70.9	0.119	0.072^{b}
SEM		0.47	0.0051	0.0054
P-values				
Temperature		<0.001	0.005	<0.001
Diet		0.134	<0.001	<0.001
Temperature × diet		0.016	0.046	0.160

[a–e]Means in the same column with no common superscript letter differ significantly ($P < 0.05$).

The effect of dietary supplementation of GAA and Arg on jejunal morphology in birds in normal or cold temperature is shown in Table 5. Villus height were affected by a temperature × diet interaction ($P \leq 0.05$), and improved in cold-stressed birds fed on a diet supplemented with 0.86 or 1.72 g/kg Arg compared to those fed on the control diet; however, no effect was observed for dietary treatments on VH under normal temperature. Dietary treatments affected ($P \leq 0.05$) crypt depth (CD), VH-to-CD ratio (VH/CD) and VW; but the main effect of temperature was only observed on VH/CD, and was higher ($P \leq 0.05$) under cold stress. Villus surface area was influenced by a temperature × diet interaction ($P \leq 0.05$). Villus surface area in cold-stressed birds fed on a diet supplemented with 1.72 g/kg Arg was improved to the extent that was similar to those grown in normal temperature, with the exception of those fed on a diet supplemented with 1.72 g/kg Arg. In addition, in cold-stressed birds GAA supplementation at 1.2 g/kg increased VSA compared to controls.

Discussion

Reports on the effect of dietary GAA supplementation on broiler performance under normal temperature are limited, and there is no report on the effect of GAA on cold-stressed birds. Earlier research had suggested that GAA supplementation at the rate of 0.6 or 1.2 g/kg led to improved performance throughout the entire production period (Ringel *et al.*, 2008; Michiels *et al.*, 2012). Furthermore, a study conducted by Mousavi *et al.* (2013) showed that the addition of GAA to the diet improved FCR of broilers receiving high-energy diets (100 and 95% of the Cobb-Vantress, Inc., recommendation) compared to low-energy diet (90%). Dilger *et al.* (2013) stated that GAA might be important in poultry nutrition in order to support overall energy homeostasis of the bird; an impact which is beyond the Arg sparing effect of GAA. In contrast, our results reveal that feeding diets supplemented with the higher level of GAA (1.2 g/kg) reduced FI of birds grown in normal temperature for the whole experimental period, with no effect of GAA under cold temperature. The reason for reduced FI in these birds is not clear; because the WG and FCR did not significantly differ compared to other treatments. Nitric oxide, produced from Arg, is a key regulator of feeding behaviour and food intake. Substances such as neuropeptide Y and ghrelin stimulate feeding through a nitric oxide pathway (Yang and Denbow, 2007). Recently, Wang *et al.* (2014) indicated that dietary Arg may regulate appetite in ducks through conversion to nitric oxide. This might explain the significantly higher FI of cold-stressed birds fed on a diet

Table 5. Effect of supplementing guanidinoacetic acid (GAA) and arginine (Arg) to the diet on jejunum morphology in broiler chickens grown in normal or cold temperatures[f]

Temperature	Diet supplementation (g/kg)	Villus height (μm)	Crypt depth (μm)	Villus height/crypt depth	Villus width (μm)	Villus surface area (mm^2)
Normal	Control	1376^{a}	255	5.44	141	0.60^{ab}
	GAA (0.6)	1325^{ab}	228	5.85	136	0.56^{bc}
	GAA (1.2)	1311^{ab}	207	6.37	144	0.59^{bc}
	Arg (0.86)	1363^{a}	218	6.27	137	0.58^{bc}
	Arg (1.72)	1407^{a}	219	6.47	150	0.66^{a}
Cold	Control	1043^{e}	235	4.45	123	0.40^{e}
	GAA (0.6)	1082de	229	4.73	128	0.43^{de}
	GAA (1.2)	1104de	224	4.93	135	0.46^{d}
	Arg (0.86)	1256^{bc}	231	5.44	135	0.53^{c}
	Arg (1.72)	1173^{cd}	226	5.24	150	0.55^{bc}
Temperature						
Normal		1356	225	6.08^{a}	142	0.60
Cold		1131	229	4.96^{b}	134	0.47
Diet supplementation (g/kg)						
Control		1209	245^{a}	4.94^{b}	132^{b}	0.50
GAA (0.6)		1204	228^{ab}	5.29^{ab}	132^{b}	0.50
GAA (1.2)		1208	216^{b}	5.65^{a}	140^{ab}	0.53
Arg (0.86)		1310	225^{ab}	5.85^{a}	136^{ab}	0.56
Arg (1.72)		1290	222^{ab}	5.86^{a}	150^{a}	0.60
SEM		35.4	9.54	0.203	5.46	0.021
P-values						
Temperature		<0.001	0.569	<0.001	0.310	<0.001
Diet		0.005	0.029	<0.001	<0.001	<0.001
Temperature × diet		0.040	0.296	0.609	0.266	0.019

[a–e]Means in the same column with no common superscript letter differ significantly ($P < 0.05$).

[f]Data represent the mean value of 12 birds (6 replicate pens × 2 birds/pen). There was one sample for jejunal segments per chick, three cross sections per sample (36 cross sections per treatment), and 10 measurements per cross section for a total of 360 measurements.

supplemented with 1.72 g/kg Arg compared to those fed on a control diet.

Supplementation of Arg reduced the negative effect of cold stress on body weight of birds and caused a significant increase in WG compared to those on control diet, without having any effect under normal temperature. Also, ascites mortality was significantly lower in birds given 0.86 g/kg Arg supplemented diets compared to control fed birds when they subjected to cold stress (7.77 vs. 3.33). In contrast, there was no difference in FCR or ascites mortality with supplementation of Arg at either level (Ruiz-Feria *et al.*, 2001). Others had reported that the growth performance of broilers raised at high altitude or high altitude plus cold temperature, which causes hypoxia, was not affected by dietary Arg supplementation compared to those fed on a control diet (Saki *et al.*, 2013; Khajali *et al.*, 2014). Furthermore, in two experiments conducted by Wideman *et al.* (1995), none of the Arg supplemented diets affected final body weights or net WG when compared to birds fed on a control diet. Inconsistent results on the effect of Arg supplementation on broiler growth performance may be attributed to the composition of the base diets to which Arg is supplemented, the total dietary level of Arg and the availability of atmospheric oxygen (Khajali *et al.*, 2014).

Broiler chickens fed on a diet supplemented with 0.86 g/kg Arg and grown in cold stress had lower RV/TV values that were comparable to those grow under normal temperature. However, no effects of Arg/GAA were observed under normal temperature. Hypoxic pulmonary vasoconstriction is counteracted by increased synthesis of the potent pulmonary vasodilator nitric oxide from Arg (Khajali *et al.*, 2013), which might have a beneficial impact on RV/TV by lowering the workload and pressure on RV. Likewise, supplementation of Arg to broiler diets significantly alleviated the adverse effect of cold stress on RV/TV (Wideman *et al.*, 1995; Tan *et al.*, 2007; Khajali *et al.*, 2014) and ascites mortality (Wideman *et al.*, 1995; Tan *et al.*, 2007). In contrast, Ruiz-Feria *et al.* (2001) did not find differences in mortality and RV/TV for cold-stressed broiler chickens fed an Arg supplemented diet compared to control fed birds.

Improved VH and VSA in birds fed on Arg supplemented diets, in comparison to control under cold temperature, might be a reason for the reduced incidence of ascites in these groups. Also, supplementation of GAA at 1.2 g/kg increased VSA in birds compared to control in the cold temperature house, but none of the supplements had any effect under normal temperature. The gastrointestinal tract is a metabolically active system that requires a large amount of oxygen (Yen *et al.*, 1989). In addition, intestinal morphology is the main indicator of gut health, and the functional status of the small intestine is characterised in part by VH and CD (Laudadio *et al.*, 2012). A lower surface area in the duodenum of birds selected for ascites susceptibility has been associated with reduced enteric function and increased incidence of ascites in these birds compared to resistant birds (Solis de los Santos *et al.*, 2005*b*). Furthermore, Solis de los Santos *et al.* (2005*a*) reported that improvement in gut morphology, and thereby efficiency, as a result of prebiotic supplementation, may reduce the negative effects of hypoxia and lead to lower ascites incidence. Improvement in jejunal morphology also might be a reason for better WG in cold-stressed birds fed on Arg supplemented diets. Comparably, Khajali *et al.* (2014) reported increased height, width and surface area of the villi in the jejunum as a consequence of Arg supplementation (10 g/kg). A more recent study suggested that addition of Arg to the culture medium stimulated growth of chicken intestinal epithelial cells (Yuan *et al.*, 2015). The proposed action of Arg in improving intestinal health includes upregulating gene expression of the target of rapamycin cell-signalling pathway that increases protein synthesis and reduces protein degradation (Yuan *et al.*, 2015).

Birds grown in cold stress on the Arg supplemented diet had lower haematocrit and H/L ratio compared to those fed on the control diet; however, neither Arg nor GAA had any effect on blood indices under normal temperature. Haematocrit (%) is an indicator of hypoxemia (Bautista-Ortega *et al.*, 2014), and a greater haematocrit value is associated with sustained hypoxia (Yersin *et al.*, 1992). In addition, a correlation exists between haematocrit values and ascites susceptibility (Wideman *et al.*, 1998). Therefore, reduced haematocrit value due to Arg supplementation might explain the lower ascites-related mortality in birds fed 0.86 g/kg Arg and raised under cold temperature compared to the control. Elevated H/L ratio indicates the presence of sustained stress (Khajali *et al.*, 2008), thus reduction of H/L ratio by Arg supplementation in the cold-stressed birds might indicate that Arg alleviates the effect of stress. Similar to the present results, haematocrit declined significantly ($P \leq 0.05$) in response to Arg supplementation (10 g/kg); but, it did not affect the H/L ratio compared to birds receiving the control diet (Khajali *et al.*, 2014). Reduced haematocrit in broilers fed supplemental Arg has also been reported by Ruiz-Feria (2009). Dobosz *et al.* (2005) reported that Arg enhances the activity of eNOS, which subsequently elevates plasma nitric oxide and reduces the haematocrit. In contrast, Wideman *et al.* (1995) reported that haematocrit values in large healthy birds were not affected by supplementation of 10 g/kg L-Arg HCl in grower or grower and finisher diets.

Kwak *et al.* (1999) suggested that Arg markedly influences lymphoid organ development in broilers, with a more pronounced effect on the thymus and spleen than the bursa of Fabricius. But, in the current experiment the temperature × diet interaction was only significant for relative bursa weight, and not spleen. Supplementing the diet with 1.72 g/kg Arg significantly reduced the weight of bursa of birds grown in either normal or cold temperature compared to birds fed on the control diet. In contrast, in the study of Deng *et al.* (2005), no differences were detected in relative thymus, spleen and bursa weights in ISA Brown commercial strain by dietary supplementation of Arg from d 1 to week 4. Carcass weight was improved in cold-stressed birds fed on a diet supplemented with 0.86 g/kg Arg compared to those fed on the control diet, with no effect of any other treatment under normal or cold temperature. In the study of Khajali *et al.* (2014), Arg supplementation did not affect carcass yield. Similarly, Mousavi *et al.* (2013) reported that the effect of GAA supplementation on carcass traits was not significant in birds grown in normal temperature, except that addition of GAA significantly ($P \leq 0.05$) reduced the relative weight of liver.

The lack of GAA effect on ascites indices or to attenuate cold stress might be due to several reasons. First, as reviewed by Hiramatsu (2003), formation of free radicals

could be promoted by guanidine compounds like GAA. Furthermore, GAA might decrease the nonenzymatic antioxidant capacity of cells (Zugno *et al.*, 2008). Based on the reports that oxidative stress is one of the main reasons in the aetiology of ascites (Akşit *et al.*, 2008), and this stress would increase in cold stress (Arab *et al.*, 2006), the proposed negative effect of GAA might have exacerbated the condition of birds grown in cold temperature in the present experiment. The results of the current study might indicate that under cold stress, the proposed negative effect of GAA might have outweighed the Arg-sparing effect of this product.

The results of this experiment indicate that unlike birds grown in normal temperature, the Arg level recommended by Ross 308 (2014) may not be adequate for cold-stressed chickens. Thus, dietary supplementation of Arg might be an effective treatment to prevent the adverse effect of cold stress on WG, jejunum morphology and ascites incidence in broiler chickens. In addition, we did not observe any beneficial effect of dietary GAA supplementation on the production performance of broilers grown in normal or subnormal temperature.

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Disclosure statement

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