

# Effects of low eggshell temperatures during incubation, in ovo feeding of L-arginine, and post-hatch dietary guanidinoacetic acid on hatching traits, performance, and physiological responses of broilers reared at low ambient temperature

Bahram Miri, Hossein Ali Ghasemi <sup>1</sup>, Iman Hajkhodadadi, and Amir Hossein Khaltabadi Farahani

*Department of Animal Science, Faculty of Agriculture and Environment, Arak University, 38156-8-8349 Arak, Iran*

**ABSTRACT** This study aimed to investigate the effect of eggshell temperature (**ET**) manipulations during incubation, in ovo feeding (**IOF**) of arginine, and post-hatch dietary supplementation with guanidinoacetic acid (**GAA**) on hatching traits and subsequent growth and physiological performance of hatched broiler chicks reared under subnormal temperature. In experiment 1, from d 8 of incubation onward, a total of 2,160 hatching eggs were randomly arranged in a 2 × 3 factorial design, in which the eggs were exposed to 2 ET (37.8°C or periodically low ET), and 3 IOF treatments (noninjected, diluent-injected, and 1% arginine solution-injected). In experiment 2, a total of 576 one-day-old male broiler chicks from 2 temperature conditions and 2 IOF treatment groups (noninjected and Arg-injected) were reared for 42 d with or without GAA supplementation in a 2 × 2 × 2 factorial design. Each treatment had 6 replicates with 12 birds each. A subnormal ambient temperature (17°C) was applied from 15 d onward to induce ascites. Results from

experiment 1 showed a 2-way interaction between ET and IOF for embryonic mortality rate during 19 to 21 d of incubation and residual yolk weight at hatch ( $P < 0.05$ ). A periodically low ET significantly increased yolk free body mass, first-grade chicks, and relative heart weight than an ET of 37.8°C. In the second experiment, overall average daily gain (**ADG**) was increased, but feed conversion ratio (**FCR**), ascites mortality, and serum thyroid hormones and corticosterone were reduced in the low ET group ( $P < 0.05$ ). There were also IOF × GAA interactions for ADG and FCR ( $P < 0.05$ ). IOF of arginine or dietary GAA increased serum nitric oxide concentration and jejunal villus height, but decreased ascites mortality ( $P < 0.05$ ). In conclusion, a periodically low ET accompanied by IOF of arginine during incubation and post-hatch dietary supplementation with GAA could be a useful strategy for improving the chick quality at hatch and subsequent improvements in post-hatch performance and ascites indices in cold-stressed broilers.

**Key words:** arginine injection, ascites, broiler embryo, eggshell temperature, guanidinoacetate

2022 Poultry Science 101:101548

<https://doi.org/10.1016/j.psj.2021.101548>

## INTRODUCTION

Genetic selection for fast growth and high meat output has resulted in reduced visceral organ development in modern broiler chickens, making them more susceptible to metabolic diseases such as ascites (Luger et al., 2001). Chicken embryos behave poikilothermally from the beginning to d 18 of incubation, which implies that their nutrient metabolism and therefore embryonic development may be affected by temperature

throughout this period (Romjin and Lokhorst, 1955). In previous investigations, the eggshell temperature (**ET**) has been found to be more important than air temperature, since the temperature of the air is simply not the same as in the embryo and may change independently, whereas the ET accounts for heat generated by both the machine and the growing embryo (Lopez et al., 2018). It has been found that an ET of 37.8°C offers the best potential for embryonic development and hatching success (Molenaar et al., 2011; Nangsuay et al., 2016; van den Brand et al., 2021). According to the literature, when embryos are exposed to high ET levels, the development of embryonic organs is slowed and the number of second-grade chicks increases (Sozcu and Ipek, 2015; Wijnen et al., 2020). However, there have been just a few researches that has addressed the impact of lower ET on hatching traits and post-hatch performance,

© 2021 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received August 30, 2021.

Accepted October 11, 2021.

<sup>1</sup>Corresponding author: [h-ghasemi@araku.ac.ir](mailto:h-ghasemi@araku.ac.ir)

especially in broiler chickens reared under cold stress conditions. In this case, [Afsarian et al. \(2016, 2018\)](#) found that periodically exposed hatching eggs to an ambient temperature of 15°C for 1 h at d 11, 13, 15, and 17 of incubation improved chick quality and development while decreasing post-hatch ascites mortality in chickens exposed to low environmental temperatures.

In ovo feeding (**IOF**) is also an effective method to improve egg nutrients and to address early development difficulties by administering exogenous nutrients (carbohydrate, protein, or amino acids) to the amnion of growing embryos of chickens at 17.5 d of incubation ([Uni et al., 2005](#); [Willemsen et al., 2010](#)). In this regard, IOF of L-arginine (**Arg**) showed beneficial impacts on post-hatch growth performance in chicks, and the effects of Arg were implicated in both glucose synthesis and hormone secretion ([Foye et al., 2006](#); [Yu et al., 2018a, b](#)). Furthermore, Arg supplementation has been shown to improve gut morphology (an indicator of gut health), which suggests that it may have an effect on the metabolism of this highly oxygen-demanding organ ([Khajali et al., 2014](#); [Gao et al., 2017](#)). It is possible that birds' Arg requirements may rise under certain conditions, such as cold stress with an increased need for oxygen ([Kodambashi Emami et al., 2017](#)). By increasing plasma nitric oxide levels, which promotes vasodilation and lowers vascular resistance, additional Arg may help to decrease ascites-related mortality ([Khajali and Wideman, 2010](#)).

Dietary guanidinoacetic acid (**GAA**), which helps with dietary Arg sparing and overall avian energy homeostasis, has also been given attention in relation to cold stress ([Nasiroleslami et al., 2018](#)). GAA is the main biologic precursor and metabolic intermediate product of creatine, which is produced from glycine and Arg in the kidney and liver of birds ([Ostojic, 2015](#)). It has been suggested that GAA is a more appropriate feed additive when compared to creatine and Arg since it is less costly than either of these compounds and is more chemically stable than creatine ([Baker, 2009](#)). Previous experiments showed that adding 0.6 g/kg of GAA to the diet of broilers reared under normal temperature conditions ([Michiels et al., 2012](#); [He et al., 2019](#)) or under heat stress ([Amiri et al., 2019](#)) led to better weight gain and feed conversion ratio. Accordingly, the inclusion of GAA in the feed, as an Arg-sparing substance, was hypothesized to enhance performance and decrease ascites mortality in broiler chickens raised under cold stress conditions.

The effect of ET modification during incubation and IOF of Arg, in conjunction with the use of post-hatch vasodilator boosting feed additives such as GAA, on thermal resistance and the occurrence of ascites in broiler chickens raised at low ambient temperatures is uncertain. Therefore, this study compared the single or combined effects of ET alteration and in ovo injection of Arg on hatchability and chick quality at hatch time, as well as the effects of combining these treatments with post-hatch dietary

supplementation of GAA on performance, blood parameters, and gut morphology of male broilers reared at suboptimal temperatures.

## MATERIALS AND METHODS

Two experiments were conducted to compare broiler chicken responses to low ET manipulation during incubation, in ovo injection of Arg, and dietary GAA supplementation when low temperatures were applied on d 15 to 42 post-hatch. All experimental procedures involving birds were complied with the "Guidelines for the Care and Use of Animals in Research" and approved by the Animal Ethics Committee of Arak University (contract number 99/2928).

### *Eggs and Incubation (Experiment 1)*

Fertile eggs of approximately similar weight ( $63.8 \pm 0.18$  g) were obtained from a commercial Ross 308 broiler breeder flock (Ross 308) at 40 wk of age ( $n = 6,500$ ), stored under commercial conditions (18°C and 65% relative humidity) for a maximum of 2 days, and then weighed. After that, the eggs that weighed within 10% of the mean weight of all the eggs were set for incubation. The eggs were subsequently transported to a multistage setter (Super J, Jamesway Incubator Company Inc., Cambridge, Ontario, Canada) in a commercial hatchery house. The eggs were incubated according to the standard temperature and humidity settings, which were monitored by an automated system. Throughout the incubation period, the eggs were rotated hourly through 90°. The experiment was designed as a  $2 \times 3$  factorial arrangement with incubation temperature group and IOF process as factors applied from d 8 to 21 of incubation. The two incubation temperature groups were the control ET group (**CONET**), in which eggs were incubated at a constant ET of 37.8°C throughout incubation, and the periodically low eggshell temperature group (**PLET**), in which eggs were subjected to a machine temperature of 15°C for 1 h on d 8, 11, 14, and 17 of incubation. Next, eggs in each temperature treatment were subjected to one of the following treatments: 1) noninjected control group (**NINJ**), 2) diluent-injected control group (**DINJ**; 0.75% NaCl solution, wt/vol), or 3) Arg solution-injected group (**AINJ**; 1% L-Arg solution, wt/vol). All injected solutions were prepared with distilled water on the day of injection. After candling at d 7 of incubation, 2,160 fertile eggs were equally divided among 6 treatment groups. Each group consisted of 10 replicate trays of 36 eggs ( $n = 10$  replicate trays/treatment group and 36 eggs/tray). The trays associated with each treatment group were randomly distributed to account for possible environmental variations induced by position in the incubator. To prepare the Arg solution, 10 g L-Arg (#A5006; Sigma-Aldrich Inc., St. Louis, MO) was dissolved in 1 L of 0.75% NaCl diluent solution to achieve a final concentration of 6 mg Arg per injection (with

injection of 0.6 mL solution). This optimal concentration of Arg injection solution was chosen based on previously reported dosage in poultry (Gao et al., 2017; Yu et al., 2018a,b). All injection solutions were made freshly and stored at 35°C for 2 h before administration on d 17.5 of incubation. The DINJ and AINJ groups were injected with 0.6 mL of solution with a 21-gauge needle inserted into the amnion at 17.5 d of incubation, which was identified by candling. The holes in the eggs were cleaned using ethyl alcohol-laden swabs and sealed with paraffin immediately after the injection, and the eggs were then transported to the incubator. In order to finish the IOF technique, all eggs were removed from their incubators for less than 30 s, and the injection process for all of the eggs, including the noninjected control eggs, was completed in less than 2 h. After injection, all eggs were transferred to hatchling basket sections within a separate 12,960-egg-capacity hatcher unit (Super J, Jamesway Incubator Company Inc.) that matched with their positioning in the setter.

### Incubation and Hatching Measurements

To monitor the ET profile during the incubation, the ET of eggs in CONET and PLET groups was measured by direct contact at the equator of the eggs using an infrared digital thermometer (IRT 6020, Braun, Kronberg, Germany) with a total of 80 eggs per treatment (8 eggs per each tray) from embryonic d 8 to 17. The hatchability rate was expressed as a percentage of fertile eggs. After hatching, all chicks were weighed and divided into 2 groups: first grade, which included those that were clean and free of lesions or deformities, and second grade, which included the rest of the chicks (Molenaar et al., 2011). A group of 40 first-grade chicks from each group ( $n = 10$  replicate trays/treatment group and 4 eggs/tray) was randomly sampled to determine the cloacal temperature. The cloacal temperatures of the chicks were measured using a digital medical thermometer (FT15/1, Beurer Company, Ulm, Germany, measurement accuracy  $\pm 0.1^\circ\text{C}$ ) that was inserted into the cloaca of the birds. These chicks from each treatment group were killed by cervical dislocation and their residual yolk, liver, and heart were weighed. The yolk free body mass (YFBM) was calculated by subtracting the body weight (BW) from the residual yolk weight. The relative weight of residual yolk was determined as the ratio of residual yolk weight to YFBM. The relative weights of liver and heart were also calculated as a ratio of live body weight (g/100 g body weight). At the end of the incubation period, all unhatched eggs were opened in order to determine the time of embryonic death, following the procedure described by Hamburger and Hamilton (1951).

### Grow-Out Phase (Experiment 2)

At hatch, chicks that had been assigned to the same treatment replicate group during the incubation phase

were pooled together and subsequently sexed and weighed. A total of 576 male chicks from 2 ET groups (CONET and PLET) and 2 IOF groups (NINJ and AINJ) were selected for a 42-d grow-out feeding period. Each of these 4 treatment groups from the incubation phase were then subdivided into 2 groups with/without dietary GAA supplementation, which resulted in a total of 8 treatments (2 incubation treatments  $\times$  2 IOF treatments  $\times$  2 GAA level). The GAA supplementation (CreAMINO, >96% GAA, Evonik Degussa GmbH, Hanau-Wolfgang, Germany) was added at a dietary level of 0.6 g/kg (Córdova-Noboa et al., 2018; Amiri et al., 2019). The birds were randomly assigned to replicate cages (6 replicates of 12 chicks per treatment) and were fed with a conventional mash broiler diet (Table 1). The diets satisfied or exceeded the strain requirements accordingly. Water and feed were supplied ad libitum from d 1 to 42 post-hatch. The temperature was kept at 33°C for the first day, and then it was gradually reduced by 3°C per week until it reached 27°C on d 14. Then, the temperature was lowered to 17°C on d 15 and maintained constant until the end of the experiment to induce ascites in chickens (Kodambashi Emami et al., 2017). The temperature was regulated with the use of a central heating system and air conditioners, followed by the use of thermostats and thermometers at 3 different room locations connected to temperature control devices. During the experimental period, the relative humidity in the rooms, which was provided by a humidifier with a capacity of 4.5 L, varied between  $55 \pm 5\%$ . A 24 h light schedule was used from d 0 to d 3, followed by a 23 h light/1 h dark schedule from d 3 until the end of the trial.

### Data Collection

Body weight and feed consumption for each experimental group were recorded on d 0, 10, 24, and 42, and then the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were determined. Mortality was taken into account when calculating the FCR by adding the BW of dead birds to the total BW of each replicate cage. The number of dead chickens was recorded daily in order to determine the mortality rate over the duration of the experiment. Mortalities due to ascites were also recorded on a daily basis, as shown by an accumulation of fluid in the abdominal cavity and pericardium, as well as an right ventricle to total ventricular ratio of more than 0.25 (Kodambashi Emami et al., 2017). The coefficient of variation of each individual BW at 42 d of age was used to determine the uniformity rate, which was obtained using the following equation (Amiri et al., 2019):

Uniformity rate

$$= 100 - [(\text{standard deviation}/\text{average BW}) \times 100].$$

On d 42, blood samples were obtained through the brachial wing vein from 2 birds in each replicate cage

**Table 1.** Ingredient composition of starter, grower, and finisher diets (as fed basis).

Ingredients (g/kg)	Starter (d 0 to 10)	Grower (d 10 to 24)	Finisher (d 24 to 42)
Corn grain	565.4	597.8	642.7
Soybean meal	299.0	266.0	222.5
Corn gluten meal	65.9	62.7	55.6
Soybean oil	18.0	27.5	36.0
Monocalcium phosphate <sup>1</sup>	15.0	13.5	12.0
Limestone	16.0	14.5	13.6
Salt (NaCl)	1.6	1.6	1.4
Sodium bicarbonate	1.9	1.9	2.3
Vitamin premix <sup>2</sup>	2.5	2.5	2.5
Mineral premix <sup>3</sup>	2.5	2.5	2.5
L-Lysine. HCL	5.1	4.3	4.1
DL-methionine	2.7	2.2	2.1
L-threonine	2.1	1.6	1.4
L-arginine	1.7	1.2	1.1
L-valine	0.6	0.2	0.2
Calculated nutritive value <sup>4</sup>			
Metabolizable energy, MJ/kg	3,000	3,100	3,200
Crude protein, g/kg	230.0	215.0	195.0
Crude fat, g/kg	43.5	53.9	63.6
Calcium, g/kg	9.6	8.7	8.0
Available phosphorus, g/kg	4.8	4.4	4.0
Digestible lysine, g/kg	12.8	11.5	10.3
Digestible methionine, g/kg	6.2	5.5	5.1
Digestible total sulfur amino acids, g/kg	9.5	8.7	8.0
Digestible threonine, g/kg	8.6	7.7	6.9
Digestible valine, g/kg	9.6	8.7	7.8
Digestible arginine, g/kg	13.7	12.3	11.0
Digestible tryptophan, g/kg	2.2	2.1	1.8
Sodium, g/kg	1.6	1.6	1.6
DEB <sup>5</sup> , mEq/100 g	250.0	235.0	220.0

<sup>1</sup>Monocalcium phosphate contained 18% Ca, 21% P.

<sup>2</sup>Supplies per kg of the diet: vitamin A (retinyl acetate), 11,000 IU; vitamin D3 (cholecalciferol), 1,800 IU; vitamin E (DL- $\alpha$ -tocopheryl acetate), 11 mg; vitamin K3 (menadione dimethylpyrimidinol), 2 mg; thiamin (thiamine mononitrate), 1.6 mg; riboflavin, 6 mg; niacin, 30 mg; D-calcium pantothenate, 15 mg; pyridoxine, 2 mg; biotin, 0.25 mg; folic acid, 0.8 mg; vitamin B12, 0.020 mg; choline (choline chloride), 500 mg.

<sup>3</sup>Supplies per kg of the diet: Mn (manganese oxide), 80 mg; Zn (zinc sulfate), 80 mg; Fe (ferrous sulfate), 35 mg; Cu (cupric sulfate), 10 mg; I (potassium iodide), 1 mg; Se (sodium selenite), 0.30 mg.

<sup>4</sup>The analyzed GAA concentrations were <1, and 624 mg/kg for 0 and 0.6 g/kg GAA-supplemented diets, respectively, during the starter period. The respective values were <1 and 579 mg/kg during the grower period, and <1 and 606 mg/kg during the finisher period Mean of two samples per diet (Evonik Industries, Evonik Degussa GmbH, Hanau-Wolfgang, Germany).

<sup>5</sup>DEB (dietary electrolyte balance) = (Na<sup>+</sup>, mEq/kg + K<sup>+</sup>, mEq/kg) – CL<sup>-</sup>, mEq/kg.

that represented the average BW of the cage. From this, 1 mL was carefully transferred into EDTA-containing tubes to assess heterophil and lymphocyte counts, while 3 mL was transferred into nonanticoagulant tubes and centrifuged at 4°C for 15 min (2,500 × *g*, 15 min) to get serum. In order to classify leukocytes, a blood smear was prepared immediately from an aliquot of whole blood from each bird and spread on microscope slides before being air-dried and fixed with methanol. After staining with May-Grunwald-Giemsa (Lucas and Jamroz, 1961), the slides were observed under an optical microscope for the differentiation of leucocyte cell counts, and 100 leucocytes were counted in each sample. The percentages of heterophils and lymphocytes, as well as the heterophil to lymphocyte ratio (**H/L**), were then determined. Serum triiodothyronine (**T<sub>3</sub>**) and thyroxine (**T<sub>4</sub>**) levels were measured by ELISA using a commercial ELISA kit (Pishtaz Teb, Tehran, Iran). The mean inter- and intra-assay CV for T<sub>3</sub> was 3.1 and 4.8%, respectively, and 4.5 and 6.1% for T<sub>4</sub>. Serum nitric oxide (**NO**) was measured by the method described by Sharifi et al. (2016).

Following blood sampling at the end of the feeding study (d 42), the chosen broiler chickens (2 chicken per replicate cage) were killed by cervical dislocation. Approximately 2 cm of the jejunum (midpoint between

the distal portion of the duodenal loop and Meckel's diverticulum) were excised, rinsed with distilled water to remove the contents, and fixed in 10% neutral-buffered formalin for histology. Transverse sections (5- $\mu$ m) were cut using a microtome and put on a glass slide, where they were stained by hematoxylin-eosin and examined under a light microscope (Olympus, CX31, Shinjuku, Tokyo, Japan). Image-analysis software (QWinPlus v. 3.1.0; Leica Cambridge Ltd.; Cambridge, UK) was used to measure villus height (**VH**; measured from the tip of the villus to the villus-crypt junction), villus width (**VW**; measured at the midpoint of the villus), and crypt depth (**CD**; measured from the depth of the invagination between adjacent villi). There were 3 cross-sections per sample (36 cross-sections for each treatment), and 10 measurements per cross-section. The VH to CD ratio was calculated using data from both the VH and the CD. In addition, the villus surface area (**VSA**) was estimated using the following equation:  $2\pi \times (VW/2) \times VH$ .

## Statistical Description

For Experiment 1, data were analyzed using the GLM procedure of SAS (SAS Institute Inc., 2010) appropriate for a  $2 \times 3$  factorial arrangement of treatments. The 2 factors were ET (CONET vs. PLET) and IOF (NINJ, DINJ, and AINJ). For Experiment 2, data were analyzed using the GLM procedure of SAS (SAS Institute Inc., 2010) appropriate for a  $2 \times 2 \times 2$  factorial arrangement of treatments. The three factors were ET (CONET vs. PLET), IOF (NINJ vs. AINJ), and dietary GAA levels (0 vs. 0.6 g/kg). The experimental unit differed in accordance with the measured parameters. In experiment 1, individual egg trays served as the experimental units for hatchability, first-grade chickens, and embryonic mortality, whereas individual chicks served as the experimental units for CT, residual yolk, YFBM, and organ weights. In experiment 2, data on growth performance parameters were analyzed on a cage basis, whereas data on blood constituents and intestinal morphology were based on individual broilers. Normality and homogeneity of variances were evaluated by Shapiro-Wilk and Levene tests, respectively. Analyses for percentage data, including hatchability, embryonic mortality, chick quality, residual yolk, and relative organ weights, were conducted after square root of arc-sine transformation of data. Total mortality and mortality due to ascites were calculated per cage and were analyzed using chi-square tests (Sozcu and Ipek, 2015). Mean separation was conducted by Tukey's post-hoc analysis with differences deemed significant at  $P < 0.05$ . The results are presented as the means with standard errors of the means.

## RESULTS

Figure 1 depicts the ET profiles during 60 min of incubation at 15°C machine temperature on d 8, 11, 14, and 17. While lowering the machine temperature on d 8 of incubation had the greatest impact on ET, it had the

least impact on ET on d 17 of incubation. The hatchability of fertile eggs, as well as the embryonic mortality rate during 0 to 3, 4 to 14, and 15 to 18 d of incubation, was not affected by experimental treatments ( $P > 0.05$ ; Table 2). However, the percentage of first-grade chicks was higher in chicks incubated at periodically low ET than at control ET ( $P < 0.05$ ). A 2-way interaction between ET and IOF was found for the embryonic mortality rate during 19 to 21 d of incubation ( $P < 0.05$ ), which was reduced with IOF of Arg in the PLET treatment.

Neither the main effects of ET and Arg injection nor their interactions on chick weight and relative liver weight were significant ( $P > 0.05$ ; Table 3). In contrast, the YFBM and relative heart weight were increased in the PLET treatment compared to the CONET treatment ( $P < 0.05$ ). The relative liver weight also tended ( $P = 0.057$ ) to be higher in the PLET group than the CONET group. As expected, the cloacal temperature of chicks at hatch in the PLET group was lower than that recorded for the CONET treatment group ( $P < 0.05$ ). IOF of Arg decreased residual yolk percentage at hatch compared with that of the NINJ and DINJ groups ( $P < 0.05$ ). An interaction was found between ET and IOF for residual yolk percentage ( $P < 0.05$ ), indicating that the response to Arg IOF was more pronounced in the CONET group than that of the PLET group.

The main effect of ET was significant for BW, ADG, FI, FCR, uniformity rate, and mortality throughout the experiment ( $P < 0.05$ ; Tables 4–6). The low ET group had 90 g greater BW on d 42 as compared with the control ET group ( $P < 0.05$ ; Table 4). From d 1 to 42, the CONET group also consumed 2.3% more feed compared with the PLET group ( $P < 0.05$ ; Table 5). The overall FCR in the PLET group was 5.4% lower than that recorded for the CONET group ( $P < 0.05$ ; Table 5). When compared to the CONET group, the PLET group showed higher uniformity rate and lower total and ascites-related mortality ( $P < 0.05$ ; Table 6). IOF of Arg at

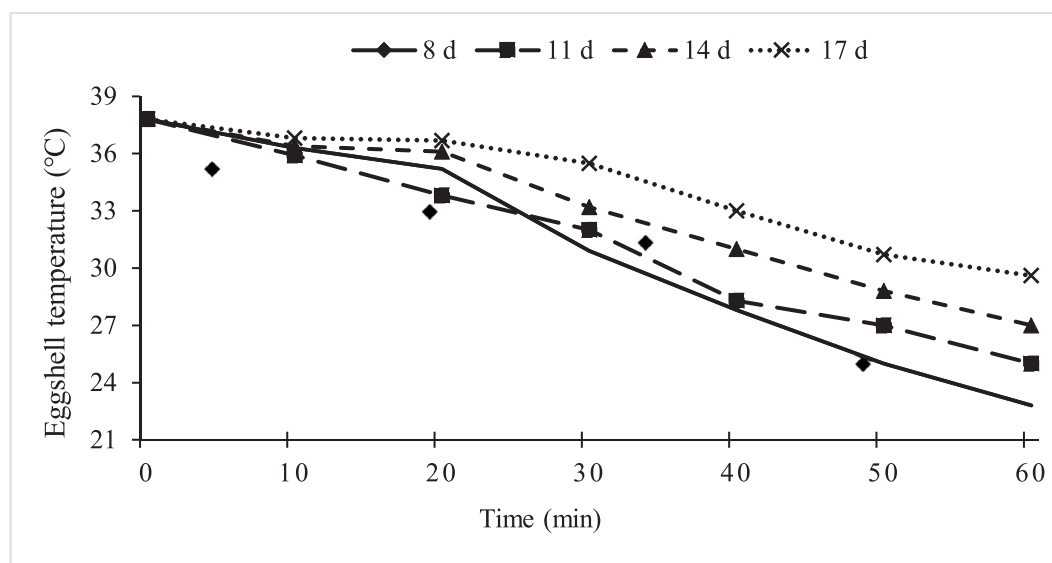


Figure 1. The eggshell temperature (ET) profiles on d 8, 11, 14, and 17 of incubation in periodical low ET treatment.

**Table 2.** Effect of periodically low eggshell temperature (ET) during incubation and in ovo feeding (IOF) of L-arginine on hatchability rate, chick quality, and embryonic mortality.

ET (°C) <sup>1</sup>	IOF <sup>2</sup>	Hatchability <sup>3</sup> , %	First-grade chicks <sup>3</sup> , %	Embryo mortality, %			
				d 0–3	d 4–14	d 15–18	d 19–21
CONET	NINJ	86.24	80.80	2.15	2.10	1.41	3.54 <sup>a</sup>
CONET	DINJ	87.03	81.17	2.11	2.09	2.04	3.87 <sup>a</sup>
CONET	AINJ	86.70	80.78	2.11	1.45	1.73	4.21 <sup>a</sup>
PLET	NINJ	86.86	83.68	2.11	2.45	2.47	4.55 <sup>a</sup>
PLET	DINJ	85.89	82.39	2.45	2.09	2.07	4.52 <sup>a</sup>
PLET	AINJ	86.63	83.51	2.12	2.45	3.17	1.04 <sup>b</sup>
SEM		1.44	1.26	0.781	0.765	0.716	0.694
Main effect of ET							
CONET		86.66	80.92 <sup>b</sup>	2.12	1.88	1.72	3.87
PLET		86.46	83.19 <sup>a</sup>	2.23	2.33	2.57	3.37
SEM		0.831	0.729	0.451	0.442	0.413	0.401
Main effect of IOF							
NINJ		86.55	82.24	2.13	2.27	1.94	4.05
DINJ		86.46	81.78	2.28	2.09	2.05	4.20
AINJ		86.66	82.15	2.11	1.95	2.45	2.62
SEM		1.018	0.892	0.552	0.541	0.506	0.491
<i>P</i> -value							
ET		0.868	0.031	0.883	0.472	0.154	0.378
IOF		0.989	0.929	0.973	0.914	0.756	0.051
ET × IOF		0.827	0.766	0.958	0.798	0.599	0.006

<sup>a,b</sup>Least square means within a column and factor lacking a common superscript differ ( $P < 0.05$ ). n: 10 replicate trays/treatment group (36 eggs/tray). Percentage data was converted to a square root arcsine transformation prior to analysis.

<sup>1</sup>CONET = eggs incubated at 37.8°C ET; PLET = eggs exposed to 15°C for 1 h on d 8, 11, 14, and 17 of incubation.

<sup>2</sup>NINJ, no injection control group; DINJ, diluent-injected control group (7.5 g NaCl dissolved in 1 L sterile distilled water), AINJ, L-arginine solution-injected group (10 g arginine dissolved in 1 L diluent).

<sup>3</sup>As a percentage of fertile eggs.

17.5 d of incubation enhanced BW, ADG, and uniformity rate, but lowered FCR and total mortality in broilers throughout the d 1 to 42 post-hatch growth-out phase ( $P < 0.05$ ). Dietary GAA supplementation also increased BW (24 and 42 d post-hatch) and ADG (d 11–24, 25–42, and 1–42), but lowered FCR (d 11–24, 25

–42, and 1–42) and overall ascites mortality ( $P < 0.05$ ). The 2-way interaction effects of ET × GAA and the 3-way interaction of ET × IOF × GAA on all performance indices were not significant ( $P > 0.05$ ; Tables 4–6). In contrast, the interactions between IOF and GAA were observed for ADG (Table 4) and FCR (Table 5) during

**Table 3.** Effect of periodically low eggshell temperature (ET) during incubation and in ovo feeding (IOF) of L-arginine on body weight (BW), yolk-free body mass (YFBM), relative organ weights, and cloacal temperature (CT) of hatchlings.

ET (°C) <sup>1</sup>	IOF <sup>2</sup>	BW, g	YFBM, g	Residual yolk <sup>3</sup> , %	Liver <sup>4</sup>	Heart <sup>4</sup>	CT, °C
CONET	NINJ	46.35	41.24	11.04 <sup>a</sup>	2.60	0.813	39.27
CONET	DINJ	46.14	41.09	10.96 <sup>a</sup>	2.59	0.850	39.32
CONET	AINJ	46.19	41.92	9.24 <sup>b</sup>	2.53	0.777	39.43
PLET	NINJ	46.53	42.06	9.60 <sup>b</sup>	2.80	0.828	38.71
PLET	DINJ	46.53	42.05	9.64 <sup>b</sup>	2.72	0.934	38.76
PLET	AINJ	46.79	42.35	9.50 <sup>b</sup>	3.01	0.922	38.56
SEM		0.329	0.389	0.367	0.169	0.045	0.140
Main effect of ET							
CONET		46.23	41.42 <sup>b</sup>	10.41 <sup>a</sup>	2.57	0.813 <sup>b</sup>	39.34 <sup>a</sup>
PLET		46.62	42.15 <sup>a</sup>	9.58 <sup>b</sup>	2.84	0.895 <sup>a</sup>	38.68 <sup>b</sup>
SEM		0.189	0.224	0.212	0.097	0.026	0.081
Main effect of IOF							
NINJ		46.44	41.65	10.32 <sup>a</sup>	2.70	0.820	38.99
DINJ		46.34	41.57	10.30 <sup>a</sup>	2.66	0.892	39.04
AINJ		46.49	42.14	9.37 <sup>b</sup>	2.77	0.849	39.00
SEM		0.232	0.275	0.260	0.119	0.032	0.098
<i>P</i> -value							
ET		0.152	0.024	0.007	0.057	0.033	<0.001
IOF		0.894	0.297	0.017	0.800	0.290	0.925
ET × IOF		0.800	0.778	0.042	0.573	0.357	0.445

<sup>a,b</sup>Least square means within a column and factor lacking a common superscript differ ( $P < 0.05$ ). n: 10 replicate trays/treatment group (36 eggs/tray for BW and 4 eggs/tray for other traits).

<sup>1</sup>CONET = eggs incubated at 37.8°C ET; PLET = eggs exposed to 15°C for 1 h on d 8, 11, 14, and 17 of incubation.

<sup>2</sup>NINJ, no injection control group; DINJ = diluent-injected control group (7.5 g NaCl dissolved in 1 L sterile distilled water), AINJ, L-arginine solution-injected group (10 g arginine dissolved in 1 L diluent).

<sup>3</sup>As a percentage of YFBM. This parameter was converted to a square root arcsine transformation prior to analysis.

<sup>4</sup>As a percentage of chick BW. The relative weights of these organs were converted to a square root arcsine transformation prior to analysis.

**Table 4.** Effect of periodically low eggshell temperature (ET) during incubation and in ovo feeding (IOF) of L-arginine with or without guanidinoacetic acid (GAA) supplementation on body weight (BW) and average daily gain (ADG) recorded from 1 to 42 d of age in broilers grown in cold temperatures.

ET (°C) <sup>1</sup>	IOF <sup>2</sup>	GAA	BW, g			ADG, g/d/bird			
			10 d	24 d	42 d	1–10 d	11–24 d	25–42 d	1–42 d
CONET	NINJ	-	208.8	934.2	2,346	16.67	51.82	78.41	54.85
CONET	NINJ	+	211.5	946.7	2,367	16.95	52.51	78.89	55.35
CONET	AINJ	-	216.3	948.8	2,383	17.42	52.32	79.66	55.73
CONET	AINJ	+	215.2	971.4	2,444	17.31	54.01	86.50	59.20
PLET	NINJ	-	217.2	955.1	2,444	17.49	52.70	82.74	57.19
PLET	NINJ	+	220.2	969.5	2,461	17.68	53.57	82.85	57.57
PLET	AINJ	-	222.4	972.1	2,467	18.04	53.55	83.07	57.75
PLET	AINJ	+	222.3	988.8	2,529	18.00	54.76	85.56	59.20
SEM			1.98	7.82	16.68	0.190	0.600	1.053	0.408
Main effect of ET									
CONET			212.9 <sup>b</sup>	950.3 <sup>b</sup>	2,385 <sup>b</sup>	17.09 <sup>b</sup>	52.67 <sup>b</sup>	80.86 <sup>b</sup>	56.28 <sup>b</sup>
PLET			220.4 <sup>a</sup>	971.4 <sup>a</sup>	2,475 <sup>a</sup>	17.80 <sup>a</sup>	53.64 <sup>a</sup>	83.55 <sup>a</sup>	57.93 <sup>a</sup>
SEM			0.99	3.91	8.34	0.095	0.300	0.527	0.204
Main effect of IOF									
NINJ			214.3 <sup>b</sup>	951.4 <sup>b</sup>	2,404 <sup>b</sup>	17.20 <sup>b</sup>	52.65 <sup>b</sup>	80.72 <sup>b</sup>	56.24 <sup>b</sup>
AINJ			219.0 <sup>a</sup>	970.3 <sup>a</sup>	2,456 <sup>a</sup>	17.69 <sup>a</sup>	53.66 <sup>a</sup>	83.70 <sup>a</sup>	57.97 <sup>a</sup>
SEM			0.99	3.91	8.34	0.095	0.300	0.527	0.204
Main effect of GAA									
-			216.2	952.5 <sup>b</sup>	2,410 <sup>b</sup>	17.40	52.60 <sup>b</sup>	80.97 <sup>b</sup>	56.38 <sup>b</sup>
+			217.1	969.1 <sup>a</sup>	2,450 <sup>a</sup>	17.49	53.71 <sup>a</sup>	83.45 <sup>a</sup>	57.83 <sup>a</sup>
SEM			0.99	3.91	8.34	0.095	0.300	0.527	0.204
<i>P</i> -value									
ET			<0.001	<0.001	<0.001	<0.001	0.028	<0.001	<0.001
IOF			0.002	0.001	<0.001	0.002	0.019	<0.001	<0.001
GAA			0.423	0.004	0.002	0.423	0.013	0.001	<0.001
ET × IOF			0.468	0.897	0.633	0.468	0.961	0.057	0.033
ET × GAA			0.836	0.863	0.921	0.836	0.822	0.122	0.071
IOF × GAA			0.227	0.576	0.078	0.227	0.431	0.005	0.001
ET × IOF × GAA			0.910	0.723	0.916	0.910	0.747	0.186	0.108

<sup>a,b</sup>Least square means within a column and factor lacking a common superscript differ ( $P < 0.05$ ). n: 6 replicate cages/treatment group (12 broilers/cage).

<sup>1</sup>CONET = eggs incubated at 37.8°C ET; PLET = eggs exposed to 15°C for 1 h on d 8, 11, 14, and 17 of incubation.

<sup>2</sup>NINJ, no injection control group; AINJ, L-arginine solution-injected group (10 g Arg dissolved in 1 L diluent).

the finisher and the entire experimental period ( $P < 0.05$ ), and the combination of Arg IOF and post-hatch dietary GAA resulted in the greatest ADG and the lowest FCR when compared to the other groups. During the period from 1 to 42 d of age, the 2-way ET × IOF interactions were also detected for overall ADG and uniformity rate ( $P < 0.05$ ), indicating that the effect of Arg IOF on these responses was more pronounced in chickens that hatched from eggs incubated at control ET.

As shown in Table 7, heterophil percentage, H/L ratio, and concentrations of serum corticosterone, T<sub>3</sub> and T<sub>4</sub> were lower ( $P < 0.05$ ), whereas lymphocyte percentage was greater ( $P < 0.05$ ) in broilers hatched from eggs incubated at periodical low ET than in those hatched from eggs incubated at control ET. The serum NO concentration of the AINJ group was greater ( $P < 0.05$ ) than that of the NIJC group ( $P < 0.05$ ). Supplemental GAA also increased ( $P < 0.05$ ) blood NO and T<sub>3</sub> concentrations in cold-stressed broiler chickens. The serum corticosterone concentration also tended ( $P = 0.064$ ) to be lower in broilers fed 0.6 g of GAA/kg of feed. However, the 2-way interaction effects of ET × IOF, ET × GAA, IOF × GAA, and the 3-way interaction of ET × IOF × GAA were not significant for all blood parameters (Table 7).

None of the morphological parameters investigated in this study were affected by ET treatment or the 2-way

interaction effects of ET × IOF, ET × GAA, IOF × GAA, or the 3-way interaction of ET × IOF × GAA ( $P > 0.05$ ; Table 8). In contrast, IOF of Arg increased VH and VH/CD of jejunum mucosa in 42-day-old broilers compared with those of the NINJ group ( $P < 0.05$ ). The inclusion of GAA also resulted in greater jejunal VH and VSA ( $P < 0.05$ ).

## DISCUSSION

Chick quality parameters have been shown to be critical for a successful start in chick and broiler production (Meijerhof, 2009). According to the results of the current study, chicks exposed to periodical cold ET during incubation had lower residual yolk weight and greater YFBW as compared with chicks under control ET conditions. During the late stages of incubation, the yolk sac is absorbed into the embryo's abdominal cavity, supplying nutrition to the chicks throughout their early post-hatch life. Embryos use the lipids from the yolk sac to initiate body growth and development of organs like the small intestine (Noy and Sklan, 1999). Therefore, it is likely that less development and growth will happen if a large quantity of residual yolk is present (Meijerhof, 2009). The decrease in ET was found to enhance the embryo's metabolic rate and heat production (van der

**Table 5.** Effect of periodically low eggshell temperature (ET) during incubation and in ovo feeding (IOF) of L-arginine with or without guanidinoacetic acid (GAA) supplementation on average daily feed intake (ADFI) and feed conversion ratio (FCR) recorded from 1 to 42 d of age in broilers grown in cold temperatures.

ET (°C) <sup>1</sup>	IOF <sup>2</sup>	GAA	ADFI, g/d/bird				FCR			
			1–10 d	11–24 d	25–42 d	1–42 d	1–10 d	11–24 d	25–42 d	1–42 d
CONET	NINJ	-	21.71	86.52	176.3	109.5	1.30	1.67	2.25	2.00
CONET	NINJ	+	21.71	86.46	175.0	109.0	1.28	1.65	2.22	1.97
CONET	AINJ	-	21.73	86.93	175.8	109.5	1.25	1.66	2.21	1.97
CONET	AINJ	+	21.66	85.53	174.5	108.5	1.25	1.58	2.02	1.83
PLET	NINJ	-	21.80	85.16	171.6	107.1	1.25	1.62	2.07	1.87
PLET	NINJ	+	21.76	85.07	169.4	106.2	1.23	1.59	2.04	1.84
PLET	AINJ	-	21.68	85.39	171.3	107.0	1.20	1.59	2.06	1.85
PLET	AINJ	+	21.75	84.66	169.3	106.0	1.21	1.55	1.98	1.79
SEM			0.243	1.167	2.271	1.007	0.015	0.025	0.035	0.022
Main effect of ET										
CONET			21.70	86.36	175.41 <sup>a</sup>	109.1 <sup>a</sup>	1.27 <sup>a</sup>	1.64 <sup>a</sup>	2.18 <sup>a</sup>	1.94 <sup>a</sup>
PLET			21.75	85.07	170.42 <sup>b</sup>	106.6 <sup>b</sup>	1.22 <sup>b</sup>	1.59 <sup>b</sup>	2.04 <sup>b</sup>	1.84 <sup>b</sup>
SEM			0.121	0.583	1.135	0.504	0.008	0.013	0.017	0.011
Main effect of IOF										
NINJ			21.75	85.80	173.09	108.0 <sup>a</sup>	1.27 <sup>a</sup>	1.63	2.15 <sup>a</sup>	1.92 <sup>a</sup>
AINJ			21.71	85.63	172.75	107.7 <sup>b</sup>	1.23 <sup>b</sup>	1.60	2.07 <sup>b</sup>	1.86 <sup>b</sup>
SEM			0.121	0.583	1.135	0.504	0.008	0.013	0.017	0.011
Main effect of GAA										
-			21.73	86.00	173.76	108.3	1.25	1.64 <sup>a</sup>	2.15 <sup>a</sup>	1.92 <sup>a</sup>
+			21.72	85.43	172.08	107.4	1.24	1.59 <sup>b</sup>	2.07 <sup>b</sup>	1.86 <sup>b</sup>
SEM			0.121	0.583	1.135	0.504	0.008	0.013	0.017	0.011
<i>P</i> -value										
ET			0.803	0.126	0.003	<0.001	<0.001	0.005	<0.001	<0.001
IOF			0.814	0.829	0.834	0.763	0.002	0.063	0.001	<0.001
GAA			0.959	0.493	0.299	0.207	0.410	0.012	0.002	<0.001
ET × IOF			0.879	0.916	0.956	0.925	0.555	0.963	0.097	0.148
ET × GAA			0.875	0.844	0.798	0.867	0.843	0.643	0.298	0.301
IOF × GAA			0.951	0.551	0.969	0.844	0.198	0.289	0.033	0.031
ET × IOF × GAA			0.811	0.837	0.965	0.888	0.968	0.676	0.268	0.234

<sup>a,b</sup>Least square means within a column and factor lacking a common superscript differ ( $P < 0.05$ ). n: 6 replicate cages/treatment group (12 broilers/cage).

<sup>1</sup>CONET = eggs incubated at 37.8°C ET; PLET = eggs exposed to 15°C for 1 h on d 8, 11, 14, and 17 of incubation.

<sup>2</sup>NINJ, no injection control group; AINJ, L-arginine solution-injected group (10 g arginine dissolved in 1 L diluent).

Pol et al., 2014; Afsarian et al., 2016). Metabolic rate of the fetus is mainly influenced by temperature because the fetus is a poikilothermic organism and has a limited capacity to control its body temperature by increasing or decreasing heat production throughout the incubation (Romjin and Lokhorst, 1955). Therefore, periodic low ET throughout incubation seems to enhance the yolk utilization and, as a result, decrease the residual yolk weight at the time of hatch. The YFBW of a chick with a lower residual yolk weight is expected to be higher, and the chick will also expend more energy as a result of a small residual yolk. The explanation for the increase in body development in the low ET may also be linked to organ development. As indicated in Table 3, the periodical low ET had a beneficial effect on the relative weights of the heart and liver. An increase in the size of these supply organs may help to accelerate the growth of the organism. Increased yolk utilization throughout incubation was found to promote the development of many organs, including, bone, muscles, and heart (van der Wagt et al., 2020). Hence, the reduced residual yolk weight reported in the present study may account for the higher heart and liver weights at the time of hatch in the low ET group. Our results also indicated that periodic cold stress throughout the incubation phase significantly increased the proportion of first-grade chicks. This finding is in keeping with previous research (Afsarian et al., 2016, 2018), suggesting that a

periodical reduction in machine temperature to decrease ET in setters led to a drop in the number of second-grade chicks.

Our present study indicated that IOF of 1% Arg increased YFBW at hatch in comparison to conventional hatchlings. The carbohydrate metabolism of avian embryos and neonates is dominated by hepatic gluconeogenesis (De Oliveira et al., 2008), and the gluconeogenic pathway is extremely active throughout embryonic development (Foye et al., 2007). In previous studies, Arg administration has been shown to boost glucose synthesis in the liver, which correlates with enhanced glucose 6-phosphatase activity at hatch (Tangara et al., 2010; Yu et al., 2018a). This could result in increased energy reserves in the liver and pectoral muscle, allowing chick embryos to grow and develop properly. Therefore, it is logical to assume that the administration of Arg into the eggs may lead to better development of the chick embryo during incubation, ultimately leading to an improvement in the yolk utilization and a consequent increase in the chick's YFBW. As shown by the interaction effect, IOF of Arg reduced residual yolk percentage at the time of hatching, and this effect was only observed in the control ET treatment. Considering that residual yolk weight in control ET treatment was greater than in low ET treatment, it can be stated that the positive effects of Arg on yolk



**Table 6.** Effect of periodically low eggshell temperature (ET) during incubation and in ovo feeding (IOF) of L-arginine with or without guanidinoacetic acid (GAA) supplementation on uniformity rate and total and ascites mortality recorded from 1 to 42 d of age in broilers grown in cold temperatures.

ET (°C) <sup>1</sup>	IOF <sup>2</sup>	GAA	Uniformity, %	Mortality <sup>3</sup>	
				Overall	Ascites
CONET	NINJ	-	81.15	19.05	13.10
CONET	NINJ	+	80.70	13.10	7.14
CONET	AINJ	-	85.43	10.71	7.14
CONET	AINJ	+	84.95	9.52	5.95
PLET	NINJ	-	85.28	8.33	4.76
PLET	NINJ	+	86.36	5.95	2.38
PLET	AINJ	-	86.00	5.95	3.57
PLET	AINJ	+	85.57	4.76	2.38
SEM			1.226	2.105	1.673
Main effect of ET					
CONET			83.06 <sup>b</sup>	13.10 <sup>a</sup>	8.33 <sup>a</sup>
PLET			85.80 <sup>a</sup>	6.25 <sup>b</sup>	3.27 <sup>b</sup>
SEM			0.613	1.053	0.837
Main effect of IOF					
NINJ			83.37 <sup>a</sup>	11.61 <sup>a</sup>	6.85
AINJ			85.49 <sup>b</sup>	7.74 <sup>b</sup>	4.76
SEM			0.613	1.053	0.837
Main effect of GAA					
-			84.47	11.01	7.14 <sup>a</sup>
+			84.40	8.33	4.46 <sup>b</sup>
SEM			0.613	1.053	0.837
<i>P</i> -value					
ET			0.003	<0.001	<0.001
IOF			0.019	0.013	0.085
GAA			0.934	0.079	0.029
ET × IOF			0.017	0.169	0.215
ET × GAA			0.653	0.552	0.454
IOF × GAA			0.658	0.323	0.215

ET × IOF × GAA 0.6700.5520.454

<sup>a,b</sup>Least square means within a column and factor lacking a common superscript differ ( $P < 0.05$ ). n: 6 replicate cages/treatment group (12 broilers/cage).

<sup>1</sup>CONET = eggs incubated at 37.8°C ET; PLET = eggs exposed to 15°C for 1 h on d 8, 11, 14, and 17 of incubation.

<sup>2</sup>NINJ, no injection control group; AINJ, L-arginine solution-injected group (10 g arginine dissolved in 1 L diluent).

<sup>3</sup>Mortality was converted to a chi-square test prior to analysis.

metabolism are better observed in control ET treatment.

Broiler growth performance after hatching is strongly influenced by the hatching conditions and quality of the chicks on the day of hatching (Lopez et al., 2018). Therefore, the improvement in the growth performance observed at 42 d post-hatch in the low ET group may be attributed to the higher YFBM present at the time of hatching in the respective group. Several studies (Zaboli et al., 2017; Al-Zghoul and El-Bahr, 2019; Su et al., 2020) have shown that in embryonic phases or in the first phase of life, metabolism, feed intake, and BW may be affected by thermostimulation. Thermal manipulation during the embryonic stage may activate the hypothalamushypophysis-thyroid axis and enhance fetal T<sub>4</sub> secretion (McNabb, 2007; Afsarian et al., 2018), which in turn controls metabolic rate and impacts the development of organs such as the gut (Akhlaghi et al., 2013). Despite the fact that studies of avian thyroid function are less comprehensive than those in mammals, thyroid hormones are well known to influence both

aspects of development (growth and differentiation/maturation) of birds (Ritchie, 2014). According to Afsarian et al. (2016), administering low ET at regular intervals during incubation may prevent subsequent increases in thyroid hormone levels in broiler chicks maintained in cold condition. As a result, a bird's performance increases if its metabolic rate does not alter while exposed to cold weather. This could be a contributing factor to the reduction in post-hatch feed intake observed in cold-stressed broiler chickens that were exposed to periodically low ET during the incubation phase. Ascites syndrome may also be prevented by ensuring that the thyroid hormone concentration does not rise. This may account for the decreased ascites mortality rate in the low ET treatment group when compared to the control ET treatment group. This result was consistent with earlier studies (Shinder et al., 2011; Shahir et al., 2012), which found that cold conditioning of embryos or post-hatch chicks was linked with a reduced incidence of ascites.

In agreement with the current study, Gao et al. (2017) reported that IOF of Arg (1%) could increase the BW of broiler chickens at 7- and 21-d post-hatch, which is similar to the results of Odutayo et al. (2020) that in ovo injection with Arg could improve post-hatch growth performance in FUNAAB-Alpha chickens. The increase in growth performance caused by Arg is not only related to the improvement in energy status, which helps to prevent the degradation of skeletal muscle, but it is also associated with the release of hormones that regulate nutrient metabolism in broilers (Yu et al., 2018a,b). In addition, our findings revealed that IOF of Arg decreased total mortality while also having a tendency to lower ascites-related mortality. This finding was in accordance with those of Kodambashi Emami et al. (2017), who reported a reduced ascites mortality in broiler chickens given 0.86 g/kg Arg supplemented diets when they were subjected to cold stress.

The present results indicate that GAA supplementation had a significant interaction with the Arg injection, suggesting a synergism effect of Arg injection and GAA supplementation on the cold tolerance acquisition in broilers. A mechanism underlying the positive effects of GAA in Arg-injected birds could be related to sparing Arg and facilitating its use for other physiological functions such as protein anabolism, cellular signaling, and hormonal release (Dilger et al., 2013; Majdeddin et al., 2019). As previously stated, the ability of GAA to effectively spare Arg is a crucial characteristic because birds do not have a functional urea cycle and are therefore incapable of synthesizing this amino acid and are hence entirely dependent on dietary supplies of this amino acid (Khajali and Wideman 2010). Moreover, this sparing effect is connected to the bird's ability to synthesize GAA in the kidneys, which would indicate a metabolic requirement for Arg in GAA production in this species (Khajali et al., 2020). Therefore, GAA addition could be particularly useful in diets for fast-growing broiler strains because of their high energy and nutrient demands for the supply of muscle creatine

**Table 7.** Effect of periodically low eggshell temperature (ET) during incubation and in ovo feeding (IOF) of L-arginine with or without guanidinoacetic acid (GAA) supplementation on blood nitric oxide (NO) concentration and stress indicators in 42-day-old broilers grown in cold temperatures.

ET (°C) <sup>1</sup>	IOF <sup>2</sup>	GAA	NO μmol/L	Heterophil (H) %	Lymphocyte (L) %	H/L	Corticosterone ng/mL	T <sub>3</sub> ng/mL	T <sub>4</sub> ng/mL
CONET	NINJ	-	24.64	31.67	59.83	0.531	13.45	1.97	21.38
CONET	NINJ	+	31.13	29.00	60.50	0.484	10.91	2.37	23.20
CONET	AINJ	-	31.33	29.00	61.33	0.478	11.79	2.19	21.02
CONET	AINJ	+	32.17	28.33	61.00	0.466	10.54	2.11	23.37
PLET	NINJ	-	24.37	26.17	63.83	0.415	10.49	1.54	18.21
PLET	NINJ	+	28.98	25.83	64.33	0.405	9.37	1.88	19.38
PLET	AINJ	-	29.54	26.83	63.67	0.425	9.61	1.73	18.39
PLET	AINJ	+	30.45	27.17	65.17	0.420	8.88	1.93	20.67
SEM			2.127	1.329	1.546	0.031	1.047	0.145	1.748
Main effect of ET									
CONET			29.82	29.50 <sup>a</sup>	60.67 <sup>b</sup>	0.490 <sup>a</sup>	11.67 <sup>a</sup>	2.16 <sup>a</sup>	22.24 <sup>a</sup>
PLET			28.33	26.50 <sup>b</sup>	64.25 <sup>a</sup>	0.416 <sup>b</sup>	9.59 <sup>b</sup>	1.77 <sup>b</sup>	19.16 <sup>b</sup>
SEM			1.063	0.665	0.773	0.016	0.523	0.072	0.874
Main effect of IOF									
NINJ			27.28 <sup>b</sup>	28.17	62.13	0.459	11.06	1.94	20.54
AINJ			30.87 <sup>a</sup>	27.83	62.79	0.447	10.20	1.99	20.86
SEM			1.063	0.665	0.773	0.016	0.523	0.072	0.874
Main effect of GAA									
-			27.47 <sup>b</sup>	28.42	62.17	0.462	11.33	1.86 <sup>b</sup>	19.75
+			30.68 <sup>a</sup>	27.58	62.75	0.444	9.93	2.07 <sup>a</sup>	21.65
SEM			1.063	0.665	0.773	0.016	0.523	0.072	0.874
<i>P</i> -value									
ET			0.329	0.004	0.002	0.002	0.007	<0.001	0.016
IOF			0.021	0.724	0.545	0.611	0.256	0.619	0.798
GAA			0.038	0.380	0.596	0.412	0.064	0.043	0.131
ET × IOF			0.857	0.163	0.762	0.282	0.819	0.480	0.737
ET × GAA			0.767	0.380	0.705	0.616	0.515	0.590	0.882
IOF × GAA			0.128	0.482	1.000	0.653	0.572	0.142	0.740
ET × IOF × GAA			0.746	0.724	0.650	0.730	0.764	0.399	0.908

<sup>a,b</sup>Least square means within a column and factor lacking a common superscript differ ( $P < 0.05$ ). n: 6 replicate cages/treatment group (2 broilers/cage).

<sup>1</sup>CONET = eggs incubated at 37.8°C ET; PLET = eggs exposed to 15°C for 1 h on d 8, 11, 14, and 17 of incubation.

<sup>2</sup>NINJ, no injection control group; AINJ, L-arginine solution-injected group (10 g arginine dissolved in 1 L diluent).

(Michiels et al., 2012). In other research, the positive effects of dietary GAA on the growth performance of broiler chickens were found, and the conversion of GAA into creatine was identified by higher blood and muscle creatine concentrations (He et al., 2019; Zhang et al., 2019). On the other hand, the Arg-sparing properties of GAA are becoming increasingly important in a variety of situations, including high altitude and temperature stress, which both raise Arg requirements (Khajali et al., 2020). In addition to being a precursor to creatine, Arg is also a precursor to nitric oxide, which regulates blood flow as a vasodilator and helps to keep the heart healthy (Khajali and Wideman, 2010). It may be useful in the prevention of pulmonary hypertension (ascites syndrome), which is a frequent issue in fast-growing broilers raised at high altitudes and/or in temperature-challenged conditions, such as those used in this research. The present research found that GAA supplementation increased circulatory NO and reduced ascites mortality, suggesting the protective effects of GAA against cold stress conditions by sparing Arg for NO synthesis.

According to the results of the current study, broilers subjected to low-ET conditions had lower H/L ratios and corticosterone levels, with a corresponding drop in thyroid hormone levels in their blood. This result is consistent with that of Afsarian et al. (2016), who found

that lowering the incubation temperature on a regular basis reduced the blood H/L ratio in broiler chickens reared at low post-hatch temperatures. Increases in corticosterone levels and the H/L ratio (as stress indicators) are believed to be indications of hypothalamic-pituitary-adrenal (HPA) axis activation (Selvam et al., 2018), which induces long-term stress (Quinteiro-Filho et al., 2012). Higher activity of the HPA axis is characterized by increased release of corticotropin-releasing hormone from the hypothalamus and, as a result, increased secretion of adrenocorticotropic hormone from the anterior pituitary, which acts on the adrenal cortex to secrete glucocorticoids, primarily corticosterone, into the bloodstream (Wang et al., 2015). A reduction in the H/L ratio and blood corticosterone levels of the low ET treatment may thus suggest that these chickens are acclimated to changes in temperatures in their growing environment.

Birds reared under low temperature conditions on a GAA-supplemented diet had significantly greater serum T<sub>3</sub> levels and tended to have lower blood corticosterone levels than the nonsupplemented group. However, IOF of Arg had no impact on stress indices under low-temperature conditions. The decrease in corticosterone levels observed in the cold-stressed birds after GAA supplementation suggests that GAA may be effective in alleviating the effects of stress. At present, the mechanism by which the GAA regulates HPA activity is not

**Table 8.** Effect of periodically low eggshell temperature (ET) during incubation and in ovo feeding (IOF) of L-arginine with or without guanidinoacetic acid (GAA) supplementation on jejunum morphology in 42-day-old broilers grown in cold temperatures.

ET (°C) <sup>1</sup>	IOF <sup>2</sup>	GAA	Villus height μm	Villus width μm	Crypt depth μm	VH/CD <sup>3</sup>	VSA <sup>4</sup> mm <sup>2</sup>
CONET	NINJ	-	1,269	137.0	254.8	5.07	0.545
CONET	NINJ	+	1,363	158.4	252.2	5.44	0.676
CONET	AINJ	-	1,370	135.5	238.7	5.87	0.584
CONET	AINJ	+	1,361	146.9	240.4	5.84	0.628
PLET	NINJ	-	1,292	141.3	234.1	5.56	0.575
PLET	NINJ	+	1,378	148.6	242.3	5.84	0.644
PLET	AINJ	-	1,381	153.3	227.5	6.16	0.665
PLET	AINJ	+	1,405	151.2	222.4	6.43	0.668
SEM			29.8	7.71	14.98	0.366	0.037
Main effect of ET							
CONET			1,341	144.5	246.5	5.56	0.608
PLET			1,364	148.6	231.6	6.00	0.638
SEM			14.9	3.86	7.49	0.183	0.018
Main effect of IOF							
NINJ			1,325 <sup>b</sup>	146.3	245.9	5.48 <sup>b</sup>	0.610
AINJ			1,379 <sup>a</sup>	146.7	232.2	6.08 <sup>a</sup>	0.636
SEM			14.9	3.86	7.49	0.183	0.018
Main effect of GAA							
-			1,328 <sup>b</sup>	141.8	238.8	5.67	0.592 <sup>b</sup>
+			1,377 <sup>a</sup>	151.3	239.3	5.89	0.654 <sup>a</sup>
SEM			14.9	3.86	7.49	0.183	0.018
<i>P</i> -value							
ET			0.271	0.452	0.166	0.097	0.257
IOF			0.014	0.944	0.205	0.025	0.313
GAA			0.025	0.088	0.957	0.392	0.022
ET × IOF			0.844	0.211	0.972	0.996	0.240
ET × GAA			0.780	0.212	0.925	0.844	0.328
IOF × GAA			0.058	0.382	0.835	0.690	0.151
ET × IOF × GAA			0.628	0.978	0.680	0.714	0.845

<sup>a,b</sup>Least square means within a column and factor lacking a common superscript differ ( $P < 0.05$ ). n: 6 replicate cages/treatment group (2 broilers/cage).

<sup>1</sup>CONET = eggs incubated at 37.8°C ET; PLET = eggs exposed to 15°C for 1 h on d 8, 11, 14, and 17 of incubation.

<sup>2</sup>NINJ, no injection control group; AINJ, L-arginine solution-injected group (10 g arginine dissolved in 1 L diluent).

<sup>3</sup>VH/CD, villus height to crypt depth ratio; VSA, villus surface area.

<sup>4</sup>VSA, Villus surface area (mm<sup>2</sup>) =  $2\pi \times (\text{Villus width}/2) \times \text{VH}$ .

fully understood. Degroot et al. (2018) found that supplementing with GAA reduced blood 3-methylhistidine levels, which is a biomarker of skeletal protein breakdown, as well as BW loss. Accordingly, it seems that GAA supplementation may help to decrease metabolic stress (i.e., protein breakdown) in broiler chickens when they are exposed to low temperatures. In this research, dietary GAA supplementation was also shown to enhance the blood level of T<sub>3</sub>. Because greater levels of adrenal cortical hormones are thought to be responsible for hypothyroid symptoms, it is possible that the decreased corticosterone level in the GAA-supplemented groups is responsible for the increased concentration of T<sub>3</sub> (Sohail et al., 2010).

It has been reported that the morphological characteristics of the small intestine mucosal surface illustrate the absorptive process and the development of the small intestine (Yang et al., 2007). According to the present findings, improvements in VH and VH/CD in birds injected with Arg at 17.5 d of incubation, as compared to control birds kept at cold temperatures, may account for the lower incidence of ascites in these groups. When compared to resistant birds, animals chosen for ascites susceptibility have a smaller surface area in the small intestine, which has been linked with impaired enteric function and an increased incidence of ascites (Solis de los Santos et al., 2005). A recent study indicated that

adding Arg to culture media promoted growth and proliferation of chicken intestinal epithelial cells (Yuan et al., 2015). Upregulation of genes in the target cell signal pathway for rapamycin, which stimulates protein synthesis while decreasing protein degradation, is hypothesized to be one mechanism through which Arg may improve intestinal health (Yuan et al., 2015). As indicated in Table 8, in the cold temperature housing, supplementation with GAA at a dose of 6 g/kg enhanced VH and VSA in broiler chickens. Although earlier studies have shown that GAA supplementation can improve intestinal morphology under normal (Ahmadipour et al., 2018), low (Kodambashi Emami et al., 2017), and high (Amiri et al., 2019) environmental temperature conditions, the exact mechanism by which this occurs is still unknown. However, this favorable impact may be linked to the beneficial effects of Arg on intestinal health, which have already been mentioned. Improvement in jejunal morphology may also be a contributing factor to improved growth performance in cold-stressed chickens receiving in ovo Arg or given GAA-supplemented diets.

In conclusion, the low ET combined with in ovo injection of Arg resulted in the lowest embryo mortality in wk 3 of incubation, which may be linked to the high YFBM of these hatchlings. Moreover, periodical low ET from d 8 of incubation onward

appeared to positively affect embryonic development, chicken quality at hatch, and post-hatch cold tolerance indicators. The results also suggest that adding 0.6 g/kg GAA to the diet of chickens *in ovo* injected with 1% Arg solution could exert an extra beneficial effect in terms of growth performance rates under post-hatch low temperature conditions. Additionally, a beneficial effect of the GAA-supplemented diet was also observed on the stress indicators and gut health of broilers (42 d) grown in low temperature conditions.

## ACKNOWLEDGMENTS

Authors of this articles are grateful to the management board of the Morgparvar Co. (Arak, Iran), especially Dr. Saeed Lotfi as the managing director of the Hatchery Unit (Morghparvar Co., Amrabad, Markazi Province, Arak, Iran), for donating eggs and the use of the Hatch facilities. We also thank the staffs of the Animal Production Experimental Center (Arak University, Arak, Iran) for their assistance with regard to bird management and sample collection during the grow-out period.

## DISCLOSURES

The authors declare that the research was performed in the absence of any financial or commercial relationships that could be construed as a potential conflict of interest.

## REFERENCES

- Afsarian, O., M. H. Shahir, A. Akhlaghi, H. Lotfolahian, A. Hoseini, and A. Lourens. 2016. Periodical low eggshell temperatures during incubation and post hatch dietary arginine supplementation: effects on performance and cold tolerance acquisition in broilers. *Poult. Sci.* 95:2427–2434.
- Afsarian, O., M. H. Shahir, A. Lourens, A. Akhlaghi, H. Lotfolahian, A. Hoseini, and N. Mousavi. 2018. Eggshell temperature manipulations during incubation and *in ovo* injection of thyroxine are associated with a decreased incidence of cold-induced ascites in broiler chickens. *Poult. Sci.* 97:328–336.
- Ahmadipour, B., F. Khajali, and M. R. Sharifi. 2018. Effect of guanidinoacetic acid supplementation on growth performance and gut morphology in broiler chickens. *Poult. Sci.* J. 6:19–24.
- Akhlaghi, A., M. J. Zamiri, Y. Jafari Ahangari, M. R. Mollasalehi, H. Shojaie, H. Atashi, B. Navidshad, A. A. Akhlaghi, and M. Dadpasand. 2013. Growth performance and intestinal morphology in broiler chickens produced from hyperthyroid breeder hens. *Anim. Prod. Sci.* 53:1046–1051.
- Al-Zghoul, M. B., and S. M. El-Bahr. 2019. Thermal manipulation of the broilers embryos: expression of muscle markers genes and weights of body and internal organs during embryonic and post-hatch days. *BMC Vet. Res.* 15:66.
- Amiri, M., H. A. Ghasemi, I. Hajkhodadadi, and A. H. Khaltabadi Farahani. 2019. Efficacy of guanidinoacetic acid at different dietary crude protein levels on growth performance, stress indicators, antioxidant status, and intestinal morphology in broiler chickens subjected to cyclic heat stress. *Anim. Feed Sci. Technol.* 254:114208.
- Baker, D. H. 2009. Advances in protein-amino acid nutrition of poultry. *Amino Acids* 37:29–41.
- Córdova-Noboa, H. A., E. O. Oviedo-Rondón, A. H. Sarsour, J. Barnes, P. Fezola, M. Rademacher-Heilshorn, and U. Braun. 2018. Performance, meat quality, and pectoral myopathies of broilers fed either corn or sorghum based diets supplemented with guanidinoacetic acid. *Poult. Sci.* 97:2479–2493.
- De Oliveira, J., Z. Uni, and P. Ferket. 2008. Important metabolic pathways in poultry embryos prior to hatch. *Worlds Poult. Sci. J.* 64:488–499.
- Degroot, A. A., U. Braun, and R. N. Dilger. 2018. Efficacy of guanidinoacetic acid on growth and muscle energy metabolism in broiler chicks receiving arginine-deficient diets. *Poult. Sci.* 97:890–900.
- Dilger, R. N., K. Bryant-Angeloni, R. L. Payne, A. Lemme, and C. M. Parsons. 2013. Dietary guanidino acetic acid is an efficacious replacement for arginine for young chicks. *Poult. Sci.* 92:171–177.
- Foye, O. T., P. R. Ferket, and Z. Uni. 2007. Ontogeny of energy and carbohydrate utilisation of the precocial avian embryo and hatching. *Avian Poult. Biol. Rev.* 18:93–101.
- Foye, O. T., Z. Uni, and P. R. Ferket. 2006. Effect of *in ovo* feeding egg white protein,  $\beta$ -hydroxy- $\beta$ -methylbutyrate, and carbohydrates on glycogen status and neonatal growth of turkeys. *Poult. Sci.* 85:1185–1192.
- Gao, T., M. Zhao, L. Zhang, J. Li, L. Yu, P. Lv, F. Gao, and G. Zhou. 2017. Effect of *in ovo* feeding of l-arginine on the hatchability, growth performance, gastrointestinal hormones, and jejunal digestive and absorptive capacity of posthatch broilers. *J. Anim. Sci.* 95:3079–3092.
- Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49–92.
- He, D., L. Yang, J. Li, B. Dong, W. Lai, and L. Zhang. 2019. Effects of guanidinoacetic acid on growth performance, creatine metabolism and plasma amino acid profile in broilers. *J. Anim. Physiol. Anim. Nutr.* 103:766–773.
- Khajali, F., A. Lemme, and M. Rademacher-Heilshorn. 2020. Guanidinoacetic acid as a feed supplement for poultry. *Worlds Poult Sci J* 76:270–291.
- Khajali, F., M. H. Moghaddam, and H. Hassanpour. 2014. An l-Arginine supplement improves broiler hypertensive response and gut function in broiler chickens reared at high altitude. *Int. J. Biometeorol.* 58:1175–1179.
- Khajali, F., and R. F. Wideman. 2010. Dietary arginine: metabolic, environmental, immunological and physiological interrelationships. *World Poult. Sci. J.* 66:751–766.
- Kodambashi Emami, N., A. Golian, D. D. Rhoads, and M. Danesh Mesgaran. 2017. Interactive effects of temperature and dietary supplementation of arginine or guanidinoacetic acid on nutritional and physiological responses in male broiler chickens. *Br. Poult. Sci.* 58:87–94.
- Lopez, J. C., L. Kitto, and R. M. Hulet. 2018. Effect of eggshell temperature on survival rate, development at hatch, and 7-day growth. *J. Appl. Poult. Res.* 27:249–252.
- Lucas, A. M., and C. Jamroz. 1961. *Atlas of Avian Hematology* 25 Agriculture Monograph. USDA, Washington, DC.
- Luger, D., D. Shinder, V. Rzepakovskiy, M. Rusal, and S. Yahav. 2001. Association between weight gain, blood parameters, and thyroid hormones and the development of ascites syndrome in broiler chickens. *Poult. Sci.* 80:965–971.
- Majdeddin, M., A. Golian, H. Kermanshahi, J. Michiels, and S. De Smet. 2019. Effects of methionine and guanidinoacetic acid supplementation on performance and energy metabolites in breast muscle of male broiler chickens fed corn-soybean diets. *Br. Poult. Sci.* 60:554–563.
- McNabb, F. M. A. 2007. The hypothalamic-pituitary-thyroid (HPT) axis in birds and its role in bird development and reproduction. *Crit. Rev. Toxicol.* 37:163–193.
- Meijerhof, R. 2009. Incubation principles: what does the embryo expect from us? Pages 106–111 in *Proc. 20th Aust. Poult. Sci. Symp.*, 9–11 February 2009.
- Michiels, J., L. Maertens, J. Buyse, A. Lemme, M. Rademacher, N. A. Dierick, and S. de Smet. 2012. Supplementation of guanidinoacetic acid to broiler diets: effects on performance, carcass characteristics, meat quality, and energy metabolism. *Poult. Sci.* 91:402–412.
- Molenaar, R., R. Hulet, R. Meijerhof, C. M. Maatjens, B. Kemp, and H. V. D. Brand. 2011. High eggshell temperatures during incubation decrease growth performance and increase the incidence of ascites in broiler chickens. *Poult. Sci.* 90:624–632.
- Nangsuay, A., R. Meijerhof, I. Van Den Anker, M. J. W. Heetkamp, V. D. S. Morita, B. Kemp, and H. Van Den Brand. 2016. Effects of

- breeder age, broiler strain, and eggshell temperature on development and physiological status of embryos and hatchlings. *Poult. Sci.* 95:666–1679.
- Nasiroleslami, M., M. Toriki, A. A. Saki, and A. R. Abdolmohammadi. 2018. Effects of dietary guanidinoacetic acid and betaine supplementation on performance, blood biochemical parameters and antioxidant status of broilers subjected to cold stress. *J. Appl. Anim. Res.* 46:1016–1022.
- Noy, Y., and D. Sklan. 1999. Energy utilization in newly hatched chicks. *Poult. Sci.* 78:1750–1756.
- Odutayo, O. J., O. M. Sogunle, O. A. Adeyemi, and A. O. Sonibare. 2020. Influence of *in ovo* arginine feeding on hatching traits and post-hatch performance of FUNAAB-Alpha chickens in two housing types. *Trop. Anim. Health Prod.* 52:2349–2357.
- Ostojic, S. M. 2015. Advanced physiological roles of guanidinoacetic acid. *Eur. J. Nutr.* 54:1211–1215.
- Quinteiro-Filho, W. M., M. V. Rodrigues, A. Ribeiro, V. Ferraz-de-Paula, M. L. Pinheiro, L. R. M. Sá, A. J. P. Ferreira, and J. Palermo-Neto. 2012. Acute heat stress impairs performance parameters and induces mild intestinal enteritis in broiler chickens: role of acute hypothalamic-pituitary-adrenal axis activation. *J. Anim. Sci.* 90:1986–1994.
- Ritchie, M. 2014. Neuroanatomy and physiology of the avian hypothalamic/pituitary axis: clinical aspects. *Vet. Clin. North Am. Exot. Anim. Pract.* 17:13–22.
- Romjin, C., and W. Lokhorst. 1955. Chemical heat regulation in the chick embryo. *Poult. Sci.* 34:649–654.
- SAS. 2010. SAS User's Guide: Statistics. Version 9.3. SAS Inst. Inc., Cary, NC.
- Selvam, R., S. Suresh, M. Saravanakumar, C. V. Chandrasekaran, and D. Prashanth. 2018. Alleviation of heat stress by a polyherbal formulation, Phytocee™: impact on zootechnical parameters, cloacal temperature, and stress markers. *Pharmacognosy Res* 10:1–8.
- Shahir, M. H., S. Dilmagani, and B. Tzschentke. 2012. Early-age cold conditioning of broilers: effects of timing and temperature. *Br. Poult. Sci.* 53:538–544.
- Sharifi, M. R., F. Khajali, and H. Hassanpour. 2016. Antioxidant supplementation of low-protein diets reduced susceptibility to pulmonary hypertension in broiler chickens raised at high altitude. *J. Anim. Physiol. Anim. Nutr.* 100:69–76.
- Shinder, D., M. Ruzal, M. Giloh, S. Druyan, Y. Piestun, and S. Yahav. 2011. Improvement of cold resistance and performance of broilers by acute cold exposure during late embryogenesis. *Poult. Sci.* 90:633–641.
- Sohail, M. U., A. Ijaz, M. S. Yousaf, K. Ashraf, H. Zaneb, M. Aleem, and H. Rehman. 2010. Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and lactobacillus-based probiotic: dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. *Poult. Sci.* 89:1934–1938.
- Solis de los Santos, F., G. Tellez, M. B. Farnell, J. M. Balog, N. B. Anthony, H. O. Pavlidis, and A. M. Donoghue. 2005. Hypobaric hypoxia in ascites resistant and susceptible broiler genetic lines influences gut morphology. *Poult. Sci.* 84:1495–1498.
- Sozcu, A., and A. Ipek. 2015. Acute and chronic eggshell temperature manipulations during hatching term influence hatchability, broiler performance, and ascites incidence. *Poult. Sci.* 94:319–327.
- Su, Y., S. Li, H. Xin, J. Li, X. Li, R. Zhang, J. Li, and J. Bao. 2020. Proper cold stimulation starting at an earlier age can enhance immunity and improve adaptability to cold stress in broilers. *Poult. Sci.* 99:129–141.
- Tangara, M., W. Chen, J. Xu, F. R. Huang, and J. Peng. 2010. Effects of *in ovo* feeding of carbohydrates and arginine on hatchability, body weight, energy metabolism and perinatal growth in duck embryos and neonates. *Br. Poult. Sci.* 51:602–608.
- Uni, Z., P. R. Ferket, E. Tako, and O. Kedar. 2005. *In ovo* feeding improves energy status of late-term chicken embryos. *Poult. Sci.* 84:764–770.
- van den Brand, H., R. Meijerhof, M. J. W. Heetkamp, I. van den Anker, M. Ooms, B. Kemp, and R. Molenaar. 2021. Interaction between eggshell temperature and carbon dioxide concentration after day 8 of incubation on broiler chicken embryo development. *Animal* 15:100223.
- van der Pol, C. W., I. A. M. Van Rooy-Reijrink, C. M. Maatjens, I. Van Den Anker, B. Kemp, and H. Van Den Brand. 2014. Effect of eggshell temperature throughout incubation on broiler hatchling leg bone development. *Poult. Sci.* 93:2878–2883.
- van der Wagt, I., I. C. de Jong, M. A. Mitchell, R. Molenaar, and H. van den Brand. 2020. A review on yolk sac utilization in poultry. *Poult. Sci.* 99:2162–2175.
- Wang, L., F. Liu, Y. Luo, L. Zhu, and G. Li. 2015. Effect of acute heat stress on adrenocorticotropic hormone, cortisol, interleukin-2, interleukin-12 and apoptosis gene expression in rats. *Biomed. Rep.* 3:425–429.
- Wijnen, H. J., H. van den Brand, A. Lammers, I. A. M. van Rooy-Reijrink, C. W. van der Pol, B. Kemp, and R. Molenaar. 2020. Effects of eggshell temperature pattern during incubation on primary immune organ development and broiler immune response in later life. *Poult. Sci.* 99:6619–6629.
- Willemsen, H., M. Debonne, Q. Swennen, N. Everaert, C. Careghi, H. Han, V. Bruggeman, K. Tona, and E. Decuyper. 2010. Delay in feed access and spread of hatch: importance of early nutrition. *Worlds Poult. Sci. J.* 66:177–188.
- Yang, Y., P. A. Iji, A. Kocher, L. L. Mikkelsen, and M. Choctt. 2007. Effects of mannanoligosaccharide on growth performance, the development of gut microflora, and gut function of broiler chickens raised on new litter. *J. Appl. Poult. Res.* 16:280–288.
- Yu, L. L., T. Gao, M. M. Zhao, P. A. Lv, L. Zhang, J. L. Li, Y. Jiang, F. Gao, and G. H. Zhou. 2018a. *In ovo* feeding of L-arginine alters energy metabolism in post-hatch broilers. *Poult. Sci.* 97:140–148.
- Yu, L. L., T. Gao, M. M. Zhao, P. A. Lv, L. Zhang, J. L. Li, Y. Jiang, F. Gao, and G. H. Zhou. 2018b. Effects of *in ovo* feeding of l-arginine on breast muscle growth and protein deposition in post-hatch broilers. *Animal* 12:2256–2263.
- Yuan, C., Y. Ding, Q. He, M. M. M. Azzam, J. J. Lu, and X. T. Zou. 2015. L-arginine upregulates the gene expression of target of rapamycin signaling pathway and stimulates protein synthesis in chicken intestinal epithelial cells. *Poult. Sci.* 94:1043–1051.
- Zaboli, G. R., S. Rahimi, F. Shariatmadari, M. A. K. Torshizi, A. Baghbanzadeh, and M. Mehri. 2017. Physiology and reproduction: thermal manipulation during pre and post-hatch on thermo-tolerance of male broiler chickens exposed to chronic heat stress. *Poult. Sci.* 96:478–485.
- Zhang, L., J. L. Li, X. F. Wang, X. D. Zhu, F. Gao, and G. H. Zhou. 2019. Attenuating effects of guanidinoacetic acid on preslaughter transport-induced muscle energy expenditure and rapid glycolysis of broilers. *Poult. Sci.* 98:3223–3232.