Performance, antioxidant status, nutrient retention and serum profile responses of laying Japanese quails to increasing addition levels of dietary guanidinoacetic acid

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ABSTRACT
In this study, the effects of different levels of dietary guanidinoacetic acid (GAA) supplementation (0, 0.6, 1.2 and 1.8 g/kg) in laying quails was evaluated in a completely randomised design comprised of four dietary treatments of six replicates each, from 56 to 154 d of age. The highest laying rate was obtained with 1.8 g/kg of GAA, but the optimum egg weight, egg mass, shell and yolk weight were observed at 1.2 g/kg of GAA. The villus height and villus height: crypt depth ratio of ileum and apparent ileal digestibility of dry matter, crude protein, ether extract and ash were linearly and quadratically affected by GAA supplementation. Maximum glutathione peroxidase and superoxide dismutase enzyme activity and total antioxidant capacity, but minimum malondialdehyde in the serum were observed at 1.2 g/kg of GAA. The highest concentrations of very low-density lipoprotein-cholesterol, total cholesterol and creatine (CRE) were observed at 1.2 g/kg of GAA, whereas the serum low-density lipoprotein-cholesterol (LDL) was at its lowest level. The CRE, nitric oxide and homocysteine concentrations were highest when GAA at 1.8 g/kg was fed, but the highest concentration of total protein and VLDL were observed at 1.2 g/kg of GAA. In conclusion, the results indicate that GAA, especially at 1.2 g/kg, could improve performance, antioxidant status and intestinal performance in laying quails.

HIGHLIGHTS
- Supplementation of GAA up to 1.8 g/kg of the laying quail diet increased egg production rate but liver betaine content decreased by inclusion of more than 1.2 g/kg of GAA.
- Supplementation of GAA at all studied levels improved nutrient digestibility while the benefits of GAA on intestinal morphology observed at inclusion levels of 0.6 and 1.2 g/kg.
- The serum and liver antioxidant status of laying quails improved by dietary addition of GAA at 0.6 and 1.2 g/kg levels.

Introduction
Guanidinoacetic acid (GAA), as a creatine precursor, has received growing attention in poultry nutrition, mainly because most of the current poultry diets formula are relying on vegetable products, which compared to animal-based ingredients are devoid of creatine (Michiels et al. 2012). Creatine and its metabolite, phosphocreatine, are used to replenish adenosine triphosphate (ATP) to prevent the energy depletion of the cells (Tossenberger et al. 2016). After the synthesis of GAA in the kidneys, through the catalytic effect of arginine: glycine amidotransferase, GAA is transferred to the liver for the conversion to creatine. GAA is methylated by S-adenosylmethionine and S-adenosylmethionine: N-guanidinoacetate methyltransferase which is the final catalyst to convert GAA into creatine. The homocysteine (HCY), that is produced as a by-product of this methylation, must either be further metabolised via transsulfuration to become cysteine or remethylated to become methionine, which acquires its methyl group from 5-methyltetrahydrofolate or betaine (Wyss and Kaddurah-Daouk 2000).

It has also been known that creatine exerts antioxidant activity in both in vitro and in vivo conditions (Lawler et al. 2002; Sestili et al. 2006; Araújo et al. 2013). For the first time, Lawler et al. (2002) showed creatine, not creatine phosphate, acts as a selective radical scavenger without any effect on radical...
oxidants such as hydrogen peroxide and tert-butylhydroperoxide. Based on the low synergistic effect of creatine with reduced glutathione, these authors suggested that creatine can act as a supportive antioxidant rather than a primary antioxidant. Also, Sestili et al. (2006) by using three different cell lines and three different toxic generating radical species, observed that creatine moderately but significantly exerts cytoprotective antioxidant activities which were dose dependent and independent of antioxidant enzymatic activities. However, Araújo et al. (2013) showed that the inclusion of creatine in the diets of rats could affect antioxidant enzymes within the liver. It has been shown that GAA may also play antioxidant and pro-oxidant properties depending on concentration and route of administration. In broiler, dietary GAA supplementation had increased liver glutathione peroxidase activity and decreased serum MDA level (Nasiroleslami et al. 2018). However, pathological accumulation of GAA can induce oxidative stress, as shown by decreased total radical-trapping antioxidant potential, total thiol content and antioxidant enzymes activity following intrastratial infusion in rats (Zugno et al. 2008).

Furthermore, GAA has a sparing effect on arginine requirements and has been proposed as an arginine alternative by Dilger et al. (2013). Some studies indicate that arginine can reduce superoxide release, lipid peroxidation and increase antioxidant capacity (Galli 2007; Petrović et al. 2008; Attia et al. 2011). Also, Duan et al. (2015) observed that increasing the arginine levels in broiler breeder diets increased egg production rate, as well as improving the antioxidant properties of serum. Sharideh et al. (2016) reported that the efficacy of GAA in broiler breeder was higher when added to low arginine diet.

To investigate the effect of GAA in breeder laying birds, Murakami et al. (2014) increased the level of GAA up to 0.18% in the diets of meat-type quail breeders, but no changes in egg production rate was observed. However, an improvement in intestinal morphology was reported in broiler chickens following dietary GAA inclusion (Ahmadipour et al. 2018). As mentioned, creatine and arginine induce different nutritional and physiological effects, but the effect of GAA supplementation in the exact recommended level of dietary arginine on desired productive traits of laying quails and their antioxidant systems are unclear. In experiment done by Murakami et al. (2014), the investigators used conventional diets with extra supplied arginine levels (15.6 vs. 12.6 g/kg). With regarding arginine level in the diet, supplying sufficient methyl donors such as methionine or betaine in accordance with that, is necessary and each failure into accomplishing can significantly impact performance (Keshavarz and Fuller 1971). Attia et al. (2019) with betaine and Attia et al. (2012, 2013) with acetic acid showed improved performance and tolerance to heat stress due to their supplementation. Therefore, the purpose of this study was to elucidate the effects of differing levels of GAA on egg production, antioxidant status, nutrient digestibility and intestinal morphology in laying Japanese quails.

Materials and methods

All procedures used in the present study were conducted according to the guidelines for animal welfare and approved by the Animal Ethics Committee of the University of Kurdistan (Sanandaj, Kurdistan, Iran).

Birds, housing and experimental diets

A total of 144 56-d-old Japanese quail birds (Coturnix coturnix japonica) were selected with similar body weight (285 ± 27 g) and egg production rate (%79.4 ± 4.1) and randomly distributed among 24 wire-floored cages (50 × 40 × 30 cm). The cages were located in an environmentally controlled room with the room temperature and relative humidity maintained between 22–24°C and 55–60%, respectively and the lighting programme was set at 16 h of light and 8 h of dark. Water and feed were provided ad libitum throughout 98 d of the study period. Birds were randomly divided into four treatment groups, each replicated six times with six birds per each replicate. Before to the onset of the experiment, all vegetable feedstuffs was analysed by NIR analyse method for protein and amino acid contents by Evonik Industries AG Laboratories (Tehran, Iran). According to feedstuffs analyses and NRC (1994), the basal diet was formulated based on corn, soybean meal and corn gluten meal, to meet or exceed laying quail nutrient specification recommendations (NRC 1994; Table 1). Guanidinoacetic acid (96% purity, Evonik Nutrition & Care GmbH, Darmstadt, Germany) was supplemented at either 0.0, 0.6, 1.2 or 1.8 g/kg of diet, in order to produce the four experimental diets. Dietary levels of arginine among all four treatments were kept constant exactly in accordance with the recommended level by using corn gluten meal.
Table 1. Composition and nutrient levels of the experimental diets.

| Item                    | g/kg as fed | 0.6 | 1.2 | 1.8 |
|-------------------------|-------------|-----|-----|-----|
| Ingredients             |             |     |     |     |
| Corn                    | 550.70      | 550.50 | 550.30 | 550.10 |
| Soybean meal (440 g/kg CP) | 325.10     | 324.70 | 324.30 | 323.90 |
| Calcium carbonate       | 55.80       | 55.80 | 55.80 | 55.80 |
| Soybean oil             | 28.50       | 28.50 | 28.50 | 28.50 |
| Corn gluten meal        | 18.60       | 18.60 | 18.60 | 18.60 |
| Dicalcium phosphate     | 12.00       | 12.00 | 12.00 | 12.00 |
| Vitamin and mineral premix* | 5.00      | 5.00  | 5.00  | 5.00  |
| Common salt             | 3.40        | 3.40  | 3.40  | 3.40  |
| α-Methionine            | 0.90        | 0.90  | 0.90  | 0.90  |
| Guanidinoacetic acid    | 0           | 0.60  | 1.20  | 1.80  |
| Calculated composition  |             |     |     |     |
| Metabolisable energy, MJ/kg | 12.14    | 12.14 | 12.14 | 12.14 |
| Crude protein           | 201.50      | 202.70 | 203.80 | 204.90 |
| Calcium                 | 25.00       | 25.00 | 25.00 | 25.00 |
| Available phosphorus    | 3.50        | 3.50  | 3.50  | 3.50  |
| Sodium                  | 1.50        | 1.50  | 1.50  | 1.50  |
| Chlorine                | 2.80        | 2.80  | 2.80  | 2.80  |
| Lysine                  | 10.20       | 10.20 | 10.20 | 10.20 |
| Methionine + cysteine   | 7.50        | 7.50  | 7.50  | 7.50  |
| Threonine               | 7.50        | 7.50  | 7.50  | 7.50  |
| Arginine                | 12.60       | 12.60 | 12.60 | 12.60 |
| Valine                  | 9.40        | 9.40  | 9.40  | 9.40  |
| Isoleucine              | 8.40        | 8.40  | 8.40  | 8.40  |
| Leucine                 | 18.30       | 18.30 | 18.30 | 18.30 |
| Analysed composition    |             |     |     |     |
| Dry matter              | 895.40      | 897.60 | 895.60 | 896.90 |
| Crude protein           | 201.60      | 203.00 | 204.30 | 205.50 |
| Ash                     | 99.00       | 100.00 | 98.00  | 99.00  |

where H is the albumen height in mm and W is the egg weight in g. The other six eggs were weighted, broken, and their albumen and yolk weights were measured for calculation of the percentage of albumen and yolk. Shell thickness was also measured by using a precise micrometer (to the nearest 0.01 mm) in three different sites of the eggshell. Egg shells were washed by distilled water and kept at room temperature for 3 d, after which the dry weight was measured and the percentage of dry eggshell of each egg was calculated.

### Slaughtering and tissue sampling

At 95 d of the experiment, chromium oxide (Cr₂O₃) was added at 0.40 g/kg of each diet. After 72 h, birds were individually weighed. The blood was sampled from the right-wing vein of birds in experimental tubes and stored at room temperature for 3 h. The clotted blood was centrifuged at 6000 × g for 10 min and the resultant sera were collected and stored at −20°C until used. Birds were killed by severing the jugular vein and immediately after slaughter, their livers and abdominal fat were dissected and weighed. The liver of two birds was collected and snap-frozen by liquid nitrogen, and stored at −70°C until analysis. Then, the ileum was ligated and separated from the other segments of the gastrointestinal tract and the contents of the distal parts were collected into a sealed bag and immediately were stored at −20°C for nutrient digestibility measurements. Also, the ileum tissue sample (2-cm segments) were taken at the midpoint of the proximal ileum and dipped in a phosphate-buffered formalin solution.

### Performance and egg quality traits

On a daily basis, the egg number and weight from each cage were recorded and were used to a weekly basis for estimation of hen-day egg production, mean egg weight and egg mass production. Average feed intake (FI, g) and feed conversion ratio (FCR, g feed per g egg mass) were measured on a weekly basis. From each replicate, 12 eggs were collected every 2 weeks and kept at room temperature for 2 d for egg quality analysis. Egg length and width were measured and egg shape index (ESI) was calculated as egg width to egg length ratio. Six eggs from each cage were weighted, broken individually on a glass plate, and the height of the albumen was measured in three different points to calculate the Haugh unit according to Brant (1951):

\[
\text{Haugh unit} = \left[100 \log \left( H + 7.57 - 1.7 W^{0.37} \right) \right]
\]

Morphological measurement of the ileal mucosa

Cross-sections of ileal segments that had been previously fixed with formalin solution were prepared using standard paraffin embedding procedures. Two sections of an intact structure at 5 μm thickness were obtained and stained with haematoxylin and eosin. The preparations were then mounted between slides and coverslips, with the addition of an aqueous agent for microscopy (Aquamount improved gun, VWR, West Chester, PA). Fifteen villi and 15 crypts of Lieberkühn from each segment of each bird were measured through the use of an optical microscope (Eclipse E600, Nikon Corp., Tokyo, Japan). The following variables were measured: villus height, crypt depth and mucosa thickness in each segment. The villus height: crypt depth ratio was calculated by dividing the villus height by the crypt depth for each observation. An
average value was calculated for each segment of each bird.

**Ileal digestibility assay**

The ileal digesta of birds was pooled and dried at 60°C for 48 h. The dried digesta and feed samples were ground, mixed, and then homogenised using a laboratory grinder (1.0-mm screen) before analysis. Dry matter was determined by drying samples of diet and ileal digesta in an oven at 103°C for 8 h. Diets and ileal digesta were analysed for nitrogen by the Kjeldahl method (AOAC 1990) for CP (N ileal digesta were analysed for nitrogen by the 6-h Soxhlet extraction. The chromium content after ashing and acid hydrolysis were analysed as described by Williams et al. (1962). Apparent ileal digestibility coefficients of dry matter, crude protein and ether extract were calculated as follows:

\[
\text{Apparent ileal digestibility (\%)} = 1 - \left( \frac{(\text{Cr}_2\text{O}_3\text{D}/\text{Cr}_2\text{O}_3\text{E}) \times (\text{N}_E/\text{N}_D)}{\text{ND}} \right) \times 100
\]

where \(\text{Cr}_2\text{O}_3\text{D}\) and \(\text{Cr}_2\text{O}_3\text{E}\) are the % chromic oxide in the diet and digesta, respectively; and \(\text{N}_E\) and \(\text{N}_D\) are the % nutrient in the digesta and diet, respectively.

**Assay of antioxidant status and metabolites in serum and liver**

The collected samples of liver were homogenised in ice-cold isotonc physiological saline, and the homogenates at the 0.1 g/mol concentration were obtained, centrifuged and the supernatants were separated. Then the serum and supernatant samples were subjected to the measurement of enzymatic activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) as well as malondialdehyde (MDA) levels using a spectrophotometer (Hitachi U-2001, Tokyo, Japan) as described in the literature. The xanthine oxidase method was used for the measurement of SOD activity which monitors the inhibition of the reduction of nitro blue tetrazolium by the sample (Winterbourn et al. 1975). The activity of GSH-Px was measured by 5,5'-dithiobis-p-nitrobenzoic acid at 412 nm by quantifying the rate of oxidation of reduced glutathione to oxidised glutathione (Hafeman et al. 1974). The MDA level was analysed with 2-thiobarbituric acid, monitoring the change of absorbance at 532 nm (Jensen et al. 1997). Furthermore, serum TAC was determined by ABTS assay as per the method of Randox kit (Pars Azmun, Tehran, Iran). The values were expressed per mL in the serum and per mg protein in the liver tissue. Protein concentrations were determined by the method of Bradford (1976) using crystalline bovine serum albumin as a standard.

Serum concentration of triglycerides, total cholesterol, low-density lipoprotein (LDL)-cholesterol, high density lipoprotein (HDL)-cholesterol, very low-density lipoprotein (VLDL)-cholesterol, creatine and creatinine were assayed using commercially available spectrophotometric kits (Sigma Aldrich Company Ltd., Poole, Dorset, UK). Serum nitric oxide (NO) concentration was determined according to Haddad et al. (1995) by adding 100 µL of the serum to 100 µL of the Griess reagent and the absorbance was measured at 540 nm using a spectrophotometer. Serum samples were screened for homocysteine using a kit (BioVision Incorporated, Milpitas, CA, USA) method based on fluorescence polarisation immunoassay.

Total lipid content of the liver was measured by homogenising a weighed slice of the liver with 5 mL chloroform/methanol 2:1 (v/v) containing 0.005% BHT and hydroquinone according to the method described by Kelley et al. (2004). A slice of the liver was minced and homogenised in 1:10 (v/v) ice-cold 50 mmol phosphate buffer (pH 7) and was used for the estimation of total protein. For betaine analysis, frozen liver samples were homogenised in 15% trichloracetic acid at 1:4 (v/v), then protein-free supernatants were subjected to exhaustive extraction and periodide complexation as described by Barak and Tuma (1979). Betaine periodide was precipitated, re-suspended in dichloroethane and analysed spectrophotometrically at 365 nm.

**Statistical analyses**

The collected data were subjected to analysis of variance by the GLM procedure as described in the SAS User’s Guide (version 9.1, SAS Institute, Cary, NC, USA). The following model was applied for analysing the results:

\[
y_{ij} = \mu + T_i + e_{ij}
\]

where \(y_{ij}\) is the value of each observation; \(\mu\) is the overall observations mean; \(T_i\) is the treatment effect and \(e_{ij}\) is the residual effect term. Tukey’s studentized-range test was used to compare difference among the treatments means at \(p < .05\). Also, the possible linear and quadratic effects of dietary GAA levels were determined by using orthogonal polynomials contrast tests. For performance characteristics, the mean of each cage was used as the experimental unit, whereas individual bird data were used for other variables and the cage was considered as random effect. Percentage
Results

Productive performance

The addition of GAA to the experimental diets had a significant effect (p < .05) on all studied performance criteria as shown in Table 2. The highest egg production (EP) rate was obtained when the basal diet was supplemented with 1.8 g/kg of GAA (linearly, p < .05). Egg weight (EW), egg mass (EM), feed consumption (FC) and feed conversion efficiency (FCE) were affected both linearly and quadratically (p < .05) upon an increasing level of dietary GAA, with the highest effect observed at the supplementation of 1.2 g/kg of GAA. Quails given 1.8 g/kg supplemental GAA showed a significant increase (p < .05) in the ratio of abdominal fat (AF) to body weight. Also, through the addition of GAA to diets, body weight change was significantly decreased (p < .05). By GAA supplementation and increasing egg production, body weight gain was decreased.

Egg quality parameters

During the experiment, no broken or abnormal eggs in any of the experimental groups were observed. The data of dietary GAA supplementation effects on egg quality parameters in laying quails are shown in Table 3. The results showed that GAA inclusion did not have significant effects (p < .05) on eggshell thickness, Haugh unit and shape index. Yolk and eggshell percentage were affected by GAA supplementation linearly and quadratically (p < .05). Yolk percentage was increased significantly (p < .05) by GAA addition up to 1.2 g/kg of diet, but then decreased by increasing the level of GAA supplementation to 1.8 g/kg. By increasing GAA supplementation, in contrast to the EP increase, the eggshell percentage was decreased significantly (p < .05). Also, albumen percentage, contrary to increasing in yolk percentage, was quadratically reduced (p < .05).

Morphological traits

Dietary GAA supplementation significantly influenced (p < .05) all morphological characteristics that were measured in this experiment (Table 4). Mucosa thickness was affected by GAA levels and had the lowest values in the diet with 1.2 g/kg GAA supplementation. Villus height and villus height: crypt depth ratio were linearly and quadratically affected (p < .05) by increasing the GAA levels in the diet, whereas crypt depth responded quadratically (p < .05) to GAA supplementation. The optimal effects were observed at 1.2 g/kg GAA supplementation.

Table 2. Effects of dietary guanidinoacetic acid (GAA) supplementation on productive traits and relative abdominal fat weight in Japanese laying quails.

| Items                              | GAA, g/kg of diet | SEM | p Value |
|------------------------------------|-------------------|-----|---------|
| Hen day production, %              |                   |     |         |
| 0                                  | 82.000b           | 86.400b | 89.800a | 91.900a | 0.866 | .010 | .010 | .170 |
| Egg weight, g                      | 11.900c           | 12.300b | 12.900a | 12.200b | 0.087 | .010 | .010 | .010 |
| Egg mass, g/bird/day               | 9.800d            | 10.600c | 11.600a | 11.200b | 0.148 | .010 | .010 | .010 |
| Feed consumption, g/bird/day       | 32.400b           | 33.000a | 33.300a | 33.200a | 0.089 | .010 | .010 | .010 |
| Feed conversion efficiency, g egg/g feed | 0.300d        | 0.320c | 0.350b | 0.340a | 0.004 | .010 | .010 | .010 |
| Initial body weight, g             | 287.500           | 285.400b | 290.800a | 286.500b | 1.040 | .450 | .780 | .590 |
| Final body weight, g               | 301.100b          | 286.300b | 289.500a | 286.100b | 2.100 | .010 | .020 | .120 |
| Body weight change, g              | 13.660b           | −0.910b | −0.360b | −1.330b | 2.030 | .100 | .010 | .580 |
| Abdominal fat, g/100 g body weight | 4.940b            | 5.130b | 5.130b | 6.000a | 0.362 | .010 | .010 | .010 |

SEM: standard errors of means.
**a-d**Means within a raw with different superscripts are significantly different (p < 0.05), Tukey’s studentized-range tests were applied to compare means.

Table 3. Effects of dietary guanidinoacetic acid (GAA) supplementation on egg quality traits in Japanese laying quails.

| Items                              | GAA, g/kg of diet | SEM | p Value |
|------------------------------------|-------------------|-----|---------|
| Eggshell thickness, mm             |                   |     |         |
| 0                                  | 0.24              | 0.25 | 0.23    | 0.24    | 0.003 | .27  | .30  | .79  |
| Shell, g/100 g egg weight          | 8.77b             | 8.67a | 8.44b | 8.39b | 0.041 | .01  | .01  | .04  |
| Yolk, g/100 g egg weight           | 30.93d            | 32.00b | 33.11a | 31.85c | 0.162 | .01  | .01  | .01  |
| Albumen, g/100 g egg weight        | 58.62a            | 58.06b | 57.66b | 58.46a | 0.095 | .01  | .08  | .01  |
| Haugh unit                         | 83.86             | 83.42 | 83.55 | 83.55 | 0.112 | .51  | .61  | .54  |
| Shape index                        | 76.13             | 75.71 | 76.72 | 76.81 | 0.681 | .46  | .87  | .19  |

SEM: standard errors of means.
**a-d**Means within a raw with different superscripts are significantly different (p < 0.05), Tukey’s studentized-range tests were applied to compare means.
**Ileal nutrient digestibility**

The data on the response of the ileal nutrient digestibility to dietary GAA supplementation are presented in Table 4. There were linear and quadratic increases ($p < .05$) in the digestibility of dry matter, crude protein, ether extract and ash through the addition of GAA to the diets.

**Serum and liver antioxidative enzyme activities and MDA contents**

The activity of SOD and GSH-Px and the levels of MDA and TAC in the serum and liver were linearly and quadratically affected ($p < .05$) by GAA supplementation to the diet (Table 5). The optimum level of enzymatic activities, serum TAC and MDA levels were obtained at 1.2 g/kg of GAA supplementation. The liver activity of GSH-Px was even lower than the control in quails which received the highest inclusion level of GAA.

**Serum and liver metabolites**

The serum and liver metabolites changes for all treatments are summarised in Table 6. Supplementation of GAA had linear and quadratic effects ($p < .05$) on creatinine, triglycerides, total cholesterol and LDL cholesterol levels in the serum of quails. Creatine, nitric oxide, homocysteine and total protein through the increase of GAA levels in the diet were linearly affected ($p < .05$). Addition of GAA to the diets induced a quadratic effect ($p < .05$) on the serum VLDL cholesterol level, and the desirable level of VLDL cholesterol was observed at 1.2 g/kg of GAA. Betaine, total protein and total lipid contents of the liver were affected quadratically ($p < .05$) by increasing GAA in the diet. In the liver, the uppermost levels of betaine and total protein were observed at 1.2 and 0.6 g/kg of GAA in the diet, respectively. The lowest level of total lipids was recognised at 1.2 g/kg dietary GAA.

**Discussion**

Commonly, corn and soybean meal are the two major components used in poultry feed, which supply arginine at levels much higher than the recommended levels. Therefore, it is necessary to consider the arginine level in the basal diets in experiments which evaluate the possible influence of GAA in poultry, as demonstrated that the response to the GAA is affected by

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**Table 4.** Effects of dietary guanidinoacetic acid (GAA) supplementation on morphological traits of the ileum and ileal digestibility of nutrients in Japanese laying quails.

| Items                        | GAA, g/kg of diet | SEM | p Value  |
|------------------------------|-------------------|-----|----------|
|                             | 0                 | 0.6 | 1.2      | 1.8      |        |
|                             | Model Linear Quadratic |
| Morphology                  |                   |     |          |          |        |
| Mucosa thickness, μm        | 242.60a           | 230.20ab | 205.00b  | 230.10ab | 7.93   | .01   | .43   | .18   |
| Villus height, μm           | 168.20b           | 187.90b | 192.60a  | 191.20a  | 2.10   | .01   | .01   | .01   |
| Crypt depth, μm             | 35.80a            | 31.80b  | 30.40b   | 34.90a   | 0.58   | .01   | .24   | .01   |
| Villus/crypt ratio          | 4.72c             | 5.92ab  | 6.39a    | 5.47b    | 0.15   | .01   | .01   | .01   |
| Digestibility               |                   |     |          |          |        |
| Dry matter, %               | 63.40b            | 69.40a  | 69.10a   | 68.50a   | 0.54   | .01   | .01   | .01   |
| Crude protein, %            | 57.30b            | 62.60a  | 63.50a   | 62.10a   | 0.57   | .01   | .01   | .01   |
| Ether extract, %            | 57.70b            | 63.00a  | 64.70a   | 62.40a   | 0.49   | .01   | .01   | .01   |
| Ash, %                      | 62.80b            | 65.10a  | 64.80a   | 64.60a   | 0.23   | .01   | .02   | .01   |

SEM: standard errors of means.

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**Table 5.** Effects of dietary guanidinoacetic acid (GAA) supplementation on antioxidant characteristics of serum and liver in Japanese laying quails.

| Items                        | GAA, g/kg of diet | SEM | p Value  |
|------------------------------|-------------------|-----|----------|
|                             | 0                 | 0.6 | 1.2      | 1.8      |        |
|                             | Model Linear Quadratic |
| Serum                       |                   |     |          |          |        |
| Superoxide dismutase, U/mL  | 2.15d             | 3.40b  | 4.12a    | 2.60c    | 0.16   | .01   | .01   | .01   |
| Glutathione peroxidase, U/mL| 1.70d             | 1.96e  | 2.85a    | 2.35b    | 0.09   | .01   | .01   | .01   |
| Total antioxidant capacity, mmol/L | 31.20d       | 36.28b | 41.73a   | 25.90d   | 1.29   | .01   | .01   | .01   |
| MDA, nmol/L                 | 5.44a             | 4.36d  | 3.14c    | 4.88b    | 0.18   | .01   | .01   | .01   |
| Liver                       |                   |     |          |          |        |
| Superoxide dismutase, U/g protein | 9.49d      | 14.23a | 12.14b   | 11.70c   | 0.35   | .01   | .01   | .01   |
| Glutathione peroxidase, U/g protein | 4.23c       | 4.89b  | 5.30a    | 3.93d    | 0.12   | .01   | .01   | .01   |
| MDA, nmol/g protein         | 13.29b           | 7.58d  | 10.15c   | 11.58b   | 0.46   | .01   | .01   | .01   |

MDA: malondialdehyde; SEM: standard errors of means.

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the level of the dietary arginine (Savage and O’Dell 1960). To assess the impacts of GAA on the performance of laying quails in the present experiment, the combination of soybean meal and corn gluten meal was used as the major protein ingredients to reduce arginine to the recommended level.

To date, there are few reports on the benefit of using of GAA as a supplement in laying quail diets, but the positive effects of other acids such as acetic acid on production performance, immunity and disease cure has been reported (Attia et al., 2012, 2013). In addition, Attia et al. (2011) reported that arginine supplementation improved immunity of chicken and tolerance to heat stress. Therefore, in the present study, more aspects of bird response to GAA supplementation was considered to obtain better insight. In the present study, by adding GAA to the diets, there was a marked increase in EP, EW and EM. The EP data suggested that quails fed 1.8 g/kg of dietary GAA had the optimum EP while the ideal level of GAA for EW and EM was 1.2 g/kg, which is in discordance with Murakami et al. (2014). They have reported no observed effects through GAA supplementation on productive traits in laying quails. These contradictions may be partly due to the use of diets with different levels of dietary arginine. As mention earlier, the chicken response to GAA and creatine supplementation is dependent to arginine concentration, such that an increase in dietary arginine level could neutralise the positive influence of GAA, mainly due to the increased necessity of transformation of GAA to creatine (Savage and O’Dell 1960).

Also, by increasing GAA supplementation, the serum nitric oxide concentration and EP was linearly, and EW and EM quadratically increased. The NO, as a metabolite of arginine, presents a fundamental function in modulating follicular growth, ovulatory mechanisms, as well as egg production in Japanese quails (Zackrisson et al. 1996; Yamauchi et al. 1997). Reduction in EW and EM at 1.8 g/kg dietary GAA was associated with the highest level of serum homocysteine and serum VLDL reduction in comparison to 1.2 g/kg GAA supplementation. Also, 1.8 g/kg GAA supplementation caused a reduction in the liver betaine and total protein levels and an increase its total lipid content. This evidence suggests that the highest level of GAA supplementation in this experiment is beyond the metabolic capacity of the liver for lipid transferring, apo-proteins and other protein synthesis, as well as homocysteine methylation. These findings could be partly explained by the findings on arginine in the literature. Silva et al. (2012) observed that through dietary arginine supplementation, egg production and egg weight of broiler breeders improved, but albumen and yolk contents, as well as eggshell percentage and thickness did not change. Also, it has reported that EP, FCR and egg weight of Xinyang Black layers reacted quadratically with increasing dietary L-arginine (Yuan et al. 2015). The increase in egg yolk that was observed in our experiment by addition of GAA to diet up to 1.2 g/kg could explain by different mechanisms. First, by sparing effect of GAA on arginine requirement for de novo creatine synthesis, more arginine is available for the synthesis of proteins which incorporate in VLDL formation and secretion. Second, as observed by Fosoul et al. (2018), partitioning of bioavailable energy tends to fat synthesis. Both of these mechanisms can be reflected in an increase of serum VLDL concentration, as observed in this experiment.

### Table 6. Effects of dietary guanidinoacetic acid (GAA) supplementation on serum and liver metabolite characteristics in Japanese laying quails.

| Items                        | GAA, g/kg of diet | SEM | Model | Linear | Quadratic |
|------------------------------|-------------------|-----|-------|--------|-----------|
| Serum                        |                   |     |       |        |           |
| Creatine, mg/dL              | 37.67 \(d\)       | 4.04 | .01   | .01    | .14       |
| Creatinine, mg/dL            | 29.40 \(d\)       | 5.28 | .01   | .01    | .14       |
| Nitric oxide, mm/l           | 4.40 \(c\)        | 5.55 | .01   | .01    | .14       |
| Hemocysteine, mm/L           | 23.52 \(c\)       | 39.24 | .01   | .01    | .14       |
| Triglycerides, mg/dL         | 53.93 \(c\)       | 40.20 | .01   | .01    | .14       |
| Total cholesterol, mg/dL     | 127.58 \(c\)      | 105.83 | .01   | .01    | .14       |
| HDL cholesterol, mg/dL       | 52.24 \(c\)       | 63.82 | .01   | .01    | .14       |
| VLDL cholesterol, mg/dL      | 72.15 \(c\)       | 56.20 | .01   | .01    | .14       |
| Total protein, g/dL          | 16.72 \(c\)       | 15.13 | .01   | .01    | .14       |
| Liver                        |                   |     |       |        |           |
| Total Lipid, mg/g fresh weight | 5.73 \(c\)   | 4.97 | .01   | .01    | .14       |
| Total protein, mg/g fresh weight | 257.00 \(c\) | 265.68 | .01   | .01    | .14       |
| Betaine, \(\mu\)g/g fresh weight | 3.18 \(c\)       | 2.87 | .01   | .01    | .14       |

HDL: high density lipoproteins; LDL: low density lipoproteins; VLDL: very low density lipoproteins; SEM: standard errors of means.

\(a-d\)Means within a raw with different superscripts are significantly different \((p < .05)\), Tukey’s studentized-range tests were applied to compare means.
In our present study, dietary treatments were isocaloric and isonitrogenous. Therefore, the increased performance with GAA supplementation may be partly attributed to improvements in the intestinal morphology, nutrient digestibility and antioxidant status. Also, feed intake significantly increased by GAA dietary supplementation at 0.6 g/kg and upward levels. Appetite is sensitive to dietary arginine deficiency and thus impairs the growth and performance in meat-type chickens (Kidd et al. 2001; Labadan et al. 2001; Attia et al., 2011) and ducks (Wang et al. 2013, 2014). Also, by inhibiting the nitric oxide synthesis, the orexigenic effects of some peptide appetite regulators (such as neuropeptide Y and ghrelin) were attenuated (Gaskin et al. 2003; Taksande et al. 2011). The increase of FI is another factor that can influence the increase in egg production in this experiment. On the other hand, an increase in nitric oxide in serum and EP suggests that, the recommended level of arginine by the NRC (1994) would be not sufficient and its revaluation is required.

High producing quails can partition dietary nutrients into egg production rather than body weight gain and maintenance. This phenomenon is evidenced in this experiment by weight reduction in high egg producing quails. However, abdominal fat percentage at 1.8 g/kg dietary GAA supplementation increased in comparison to other treatments. This might be due to an increase in energy intake caused by higher GAA concentration and the possible better efficiency of energy utilisation. Fosoul et al. (2018) observed an increase in net energy for production (NE\textsubscript{p}) with the inclusion of GAA to the diets of broiler chickens and this led in an increase in energy retention as fat.

The digestive secretions, epithelial absorptive surface area and permeability of the intestinal mucosa are the main factors which all jointly can affect the extent of intestinal nutrient uptake (Casparry 1992; Choct 2009; Hajiaghapour and Rezaeipour 2018). In addition, because the mucosal layer is a vast surface of absorptive epithelial cells that transport nutrients into the enterocytes, the minute changes in the microscopic characteristics of the mucosal layer can affect the efficiency of nutrient digestibility and uptake, feed efficiency, and performance of the birds (Choct 2009). Although it has been suggested by reducing the intestinal thickness, nutrient demand of the gastrointestinal system decreased and nutrient absorption promoted (Visek 1978). Besides, the villus height can considerably affect the digestive and absorptive roles of the intestine (Casparry 1992; Attia et al. 2013) and eventually improves the bird performance. On the other hand, crypt as villus cell producer, by a decrease in depth, shows a lower villus cell regeneration and lower metabolic demand (Yason et al. 1987). However, information on the effects of the GAA supplementation on intestinal morphology of quail is limited and most of the studies, examine the effects of arginine on gut morphology and function of other poultry. Through arginine supplementation in broiler chickens, an improvement in the duodenum morphology characteristics and jejunal and ileal villus height and absorptive surface area were reported (Khajali et al. 2014; Murakami et al. 2014). In agreement with a recent study done by Ahmadipour et al. (2018), our results showed improvements in the villus height and absorptive surface area and a decrease in crypt depth in the ileum by GAA supplementation. Arginine, in addition to its nutritional importance in growth and intestinal development (Löser et al. 1999), through the hydrolysis into urea and ornithine by kidney arginase, is involved in polyamine biosynthesis, which consecutively induces cell proliferation, protein expression, and tissue maturation (Pegg and McCann 1982). Yuan et al. (2015) also reported that arginine supplementation in broiler diets caused an upregulation of the genetic expression of the TOR (target of rapamycin) cell signalling. This increase in intestinal epithelial cells protein synthesis and reduce in the protein degradation can reduce the excretion of endogenous nitrogen compounds which therefore may increase the nutrients available to other organs to increase bird performance.

In the present study, by adding GAA to experimental diets, a significant increase in the crude protein, ether extract, ash and dry matter digestibility was observed. These results that indirectly show the functional properties of arginine and creatine can explain the improvement in morphological and functional characteristics of the intestine and thereby improve nutrient digestion and the absorptive capacity of the gastrointestinal tract.

Moreover, the antioxidant status of the whole-body system can modulate the optimum functioning of the body and improve the performance of birds. Within all cell types, including intestinal epithelial cells, hydrogen peroxide is constantly generated and causes a diverse range of gastrointestinal injury (Placha et al. 2014). Any factor that can affect the gastrointestinal health can assuredly affect the animal as a whole, and ultimately change the intestinal nutrient uptake and requirements (Choct 2009). GAA supplementation up to 1.2 g/kg of the diet increased the creatine level in the serum, which through its reactive oxygen...
scavenger property, increases the antioxidant capacity of serum (Lawler et al. 2002). Also, the increase in GSH-Px and SOD activity and a decrease in MDA level in the liver and serum implies that the antioxidant status of the bird as a whole is improved. The reduction in GSH-Px activity of the liver and TAC of serum coincided with the highest serum HCY concentration and the lowest level of betaine in the liver. Supplementation of 1.8 g/kg GAA in the diets caused an accumulation of homocysteine in the serum and liver and resulted in a reduction of the available cysteine for glutathione synthesis. Studies that investigate the effect of GAA on antioxidant systems in poultry are limited. Recently, in agreement with our results, Nasiroleslami et al. (2018) reported an increase in liver GSH-Px activity and a reduction in serum MDA content as the addition level of GAA increased to 1.2 g/kg of broiler diet. Also, by 0.8, 1.2 and 2 g/kg of GAA supplementation in pig diet, Wang et al. (2012) observed a quadratic increase in antioxidant enzymes activity. However, Duan et al. (2015) observed that supplementing of different levels of arginine in late laying broiler breeders in late periods of laying cycle, resulted in a decline of GSH-Px activity and TAC levels in serum and egg yolk, especially when arginine was supplemented at more than 1.36% of the diet. In our study, the layer quails receiving the arginine level recommended by NRC (1994) showed the lowest antioxidant capability, which might be attributed to lower serum concentration of creatine in these birds. Lastly, at the highest level of GAA supplementation, homocysteine and methyl group demand may be determinative of GAA function and efficiency.

Conclusions

It is concluded that diets supplemented by GAA at levels of 1.8 g/kg can increase egg production; and egg weight, and egg mass can be improved at 1.2 g/kg without any adverse effects on the external characteristics of the egg. These improvements can be attributed to the increase in serum NO and creatine as well as the improvement in morphological characteristics of the small intestine, resulting in improved nutrient digestibility. In addition, the increase in methyl demand at 1.8 g/kg GAA supplementation can limit the beneficial effects of the GAA.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Ethical approval

All experimental procedures were conducted according to the international protocols and approved by Research Committee of University of Kurdistan, IRAN.

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