



DSPACE

<https://dspace.org/>

Dietary L-glutamine affects eggshell quality in the post-peak laying period*

Muszyski, Siemowit; Tomaszewska, Ewa; Arczewska-Wosek, Anna; Kasperek, Kornel; Batkowska, Justyna ...

2022

<https://hdl.handle.net/10539/50252>



DIETARY L-GLUTAMINE AFFECTS EGGSHELL QUALITY IN THE POST-PEAK LAYING PERIOD*

Siemowit Muszyński¹, Ewa Tomaszewska^{2*}, Anna Arczewska-Włosek³, Kornel Kasperek⁴, Justyna Batkowska⁴, Krzysztof Lamorski⁵,
Dariusz Wiącek⁵, Janine Donaldson⁶, Sylwester Świątkiewicz³

¹Department of Biophysics, Faculty of Environmental Biology, University of Life Sciences in Lublin, Lublin, Poland

²Department of Animal Physiology, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Lublin, Poland

³Department of Animal Nutrition and Feed Science, National Research Institute of Animal Production, 32-083 Balice n. Kraków, Poland

⁴Institute of Biological Basis of Animal Production, Faculty of Animal Sciences and Bioeconomy, University of Life Sciences in Lublin, Lublin, Poland

⁵Bohdan Dobrzański Institute of Agrophysics of the Polish Academy of Sciences, Lublin, Poland

⁶School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Parktown, Johannesburg, South Africa

*Corresponding author: ewaRST@interia.pl

Abstract

In the current study the hypothesis that 1.0% dietary inclusion of glutamine (Gln), a conditionally essential amino acid that influences protein synthesis and shows anti-osteoporotic effect, can influence eggshell quality was tested on laying hens in the post-peak laying period. Thirty-week-old Bovans Brown hens were randomly assigned to control group or group supplemented with Gln in the form of alpha-ketoglutarate (10 g/kg) with 12 replicate cages (2 hens/cage) in each group. The experimental period lasted for 30 weeks, from the 31st to the 60th week of age of hens, when eggs were collected and selected eggshell quality indices were determined. While Gln supplementation had no effect on egg geometry and eggshell cracking force, the analysis of eggshell quality showed an increase of the eggshell thickness ($P<0.01$), porosity ($P<0.01$), eggshell specific surface ($P<0.001$) and positive changes in other mechanical parameters (decrease of eggshell stiffness and Young's modulus, $P<0.05$ and $P<0.01$, respectively; increase of work needed to crack the eggshell, $P<0.05$). Changes in eggshell mineral composition, including an increase of Ca content ($P<0.001$), were also observed. In conclusion, the current study showed a beneficial effect of Gln on the eggshell thickness, calcification, and some mechanical parameters.

Key words: glutamine, amino acid, laying hen, eggshell quality, eggshell cracking strength, eggshell mineralization

Egg quality, as a general term, refers to several features defining both its internal and external quality. External egg quality is focused on the eggshell, which plays an important role in poultry production, where economic losses can result from problems with regards to the eggshell, between laying and packing, including cracks caused by excess pressure, thin eggshells or shell-less eggs. A poorly formed eggshell also does not prevent the translocation of microorganisms into the egg, which can be dangerous for consumers or compromise embryo development. Additionally, the eggshell is the main source of calcium for the developing bird and it participates in the exchange of oxygen and carbon dioxide during development. The quality of eggshells is primarily influenced by the genetic origin of the laying hens, as well as the laying level, health status, housing conditions, age, and nutrition of the hens (Soltan, 2008; Sokołowicz et al., 2018; Orczewska-Dudek et al., 2020; Świątkiewicz et al., 2020). Laying hens of modern hybrid strains are

now capable of producing approximately 500 eggs in a 2-year-long life cycle. During the whole laying period hens are in a state of imbalance with regards to calcium homeostasis. Medullary bone, characteristic only for laying birds, is a highly reactive secondary bone tissue which starts forming in the marrow cavity about two weeks before they start laying and its primary role is to supply calcium for the mineralization of eggshell (Nys and Le Roy, 2018).

L-glutamine (Gln) is considered a non-essential amino acid from the point of view of physiological role in general metabolism, including protein and lipid synthesis (Burrin and Stoll, 2009; Wu et al., 2016; Xiao et al., 2016), however, it is suggested that in some critical conditions (stress, malnutrition, rapid growth or production) it can act as conditionally essential amino acid, especially in growing or stressed poultry species. Interestingly, among the doses used, ranging from 0.5% to 4.0%, it was shown that performance indices, intestinal morphology,

*This work was supported by Statutory Research of the Department of Animal Physiology, University of Life Sciences in Lublin, no. WKF/S/32/2020 and WKF/S/32/2021.

humoral immune response, and blood morphology are optimized for all poultry species following 1.0% (10 g/kg) Gln supplementation (Sakamoto et al., 2006; Bartell and Batal, 2007; Soltan, 2009; Zavarize et al., 2011; Jazideh et al., 2014; Xiao et al., 2014; Luquetti et al., 2016; Xue et al., 2018). In egg-laying poultry, 1.0% Gln has the most positive effects on egg quality traits and fertility parameters in guinea fowls as reported by Gholipour et al. (2007, 2019), while Zavarize et al. (2011) showed that 1.0% Gln improved feed intake, feed conversion per kilogram of eggs, and egg quality in laying hens. What is more, a long-term 1.0% supplementation of Glu in the form of α -ketoglutaric acid (AKG) was especially effective in improving the microstructure of trabeculae of medullary bone (Tomaszewska et al., 2020). Thus, it can be speculated that as the eggshell formation depends on the medullary bone quality and the function of intestine, Glu supplementation can affect also the eggshell quality.

Therefore we hypothesized that 1.0% Gln supplementation may improve eggshell properties due the dependence of eggshell formation on medullary bone metabolism. The objective of study was to verify the hypothesis presented above and investigate whether Gln given to hens at a dose of 1.0% for 30 weeks in the middle birds' peak lay period, could improve eggshell quality.

Material and methods

The study was carried out in compliance with the EU Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific or educational purposes and in compliance with the ARRIVE guidelines.

The experiment was carried out using 48 Bovans Brown hens obtained from a local commercial source. Hens arrived at the age of 17 weeks and for 13 weeks of pre-experimental period were fed the same standard commercial pre-laying (18–20 weeks) and laying (21–30 weeks) diets. Hens were housed at the rate of two birds per cage in wire-mesh floor cages (30×120×50 cm), under controlled climate conditions ($19 \pm 2^\circ\text{C}$; 50–65% RH; 14 L:10 D lighting schedule with light intensity of 10 lux), with free access to feed and water. At the age of 31 weeks, hens were randomly assigned to one of the two groups, each comprising $n=12$ replicate cages, with two birds per cage. The control group was fed the basal diet (Table 1) and the experimental group (the Gln group) was fed the basal diet plus 1.0% (10 g/kg) of Gln in the form of α -ketoglutaric acid (AKG). The dose selection was based on the data from the literature, being the most effective for poultry species as reported by EFSA (EFSA, 2020). All the diets (pre-experimental and experimental) met the nutrient requirements specified by the Polish recommendations (Smulikowska and Rutkowski, 2018). The chemical composition of the basal feed was analyzed according to EU Commission Regulation 152/2009 (EU, 2009). Gln was administered “on top” of the complete

diet and was not taken into account when balancing the compound feed.

Table 1. Composition (g/kg) and nutritive value (g/kg) of the basal diet (air-dry basis)

Item	Content
Corn	422.10
Wheat	210.00
Soybean meal	236.00
Rapeseed oil	20.00
Limestone	90.00
Monocalcium phosphate	12.50
NaCl	3.00
DL-methionine	1.40
Premix vit./min ¹	5.00
Metabolizable energy (MJ/kg) ²	11.60
Crude protein ³	170.00
Lysine ³	8.35
Methionine ³	4.1
Methionine + Cysteine ³	7.2
Tryptophan ³	2.0
Threonine ³	6.3
Isoleucine ³	4.5
Leucine ³	7.1
Valine ³	14.5
Phenylalanine + Tyrosine ³	8.0
Total calcium ³	37.00
Total phosphorus ³	6.15
Available phosphorus ³	3.90

¹The premix provided per 1 kg of diet: vitamin A, 10,000 IU (retinol); vitamin D₃, 3,000 IU (cholecalciferol); vitamin E, 50 IU (dl- α -tocopherol); vitamin K₃, 2 mg (menadione); vitamin B₁, 1 mg (thiamine); vitamin B₂, 4 mg (riboflavin); vitamin B₆, 1.5 mg (pyridoxine); vitamin B₁₂, 0.01 mg (cyanocobalamin); Ca-pantothenate, 8 mg; niacin, 25 mg; folic acid, 0.5 mg; choline chloride, 250 mg; manganese, 100 mg; zinc, 50 mg; iron, 50 mg; copper, 8 mg; iodine, 0.8 mg; selenium, 0.2 mg, cobalt, 0.2 mg.

²Calculated as a sum of the ME content of components.

³Calculated value from the chemical composition of raw feedstuffs.

The experimental period covered the middle of the post-peak laying period and lasted for 30 weeks until the hens reached the age of 60 weeks. On the last two days of the 30-week experimental period, two eggs from each cage were randomly collected and stored in a cold room (5°C) for no longer than for 3 days after collection. The values of each measured trait of an individual egg in a replicate cage were averaged per cage prior to statistical analysis.

Measurements of basic egg external quality indices

Egg weight and specific gravity (based on dry and wet egg weight measurements, according to Archimedes principle) were determined. The shape of the egg (long and short axis length) was measured using vernier caliper and the shape index (the ratio of long and short

axis length), geometric mean diameter (Mohsenin, 1986; Altuntas and Sekeroglu, 2008), volume and surface area (Narushin, 2005) were calculated. Eggshell color was expressed as a percentage of reflected light (Drabik et al., 2020). The eggshell thickness was measured at 45° from the large end of the egg using an ultrasonic eggshell thickness gauge (ESTG-1, Orka Food Technology Ltd, Ramat Hasharon, Israel) (Kibala et al., 2015).

Measurements of eggshell mechanical properties

Eggshell mechanical properties were measured with an Instron Mini 55 apparatus (American Instrument Exchange, Haverhill, MA, USA). Eggs were compressed at a constant loading rate of 50 mm/min until the eggshells cracked. Eggshell structural properties were determined from the recorded load-displacement curves using Origin software (ver. 2016, OriginLab, Northampton, MA, USA). The cracking force was determined as the force that caused the egg to crack. Stiffness was measured as the slope of the elastic part of the load-displacement curve (Xiao et al., 2014). Firmness was determined as the ratio of cracking force to the absolute value of deformation (Altuntas and Sekeroglu, 2008) and the total work to eggshell cracking was determined as the total area under the load-displacement curve. Since the structural properties of the eggshell do not describe the physical properties of the eggshell material, intrinsic material properties of the eggshell material were also calculated, using formulas developed by Bain (1990). Stiffness, radius of curvature, the eggshell thickness and shape index were used to calculate the elastic Young's modulus (Mabe et al., 2003). Fracture toughness was calculated based on measurements of cracking force, radius of curvature and shell thickness (Mabe et al., 2003).

Measurements of eggshell quality indices

The number of eggshell pores was determined according to Tyler (1953) and Roudybush et al. (1980). Briefly, after boiling in 2.5% NaOH (to remove protein fibers) and etching in concentrated 65% nitric acid, eggshell samples were washed in distilled water and dried. After drying, a 0.01% aqueous solution of methylene blue was applied to the inner surface of the shell to make the pores visible, then samples were washed again using distilled water. For each eggshell, three microscopic images of three randomly chosen areas were recorded using a stereoscopic microscope (SZX16, Olympus, Tokyo, Japan). Pores were counted using ImageJ software (NIH, Bethesda, MA, USA) on three randomly selected areas for each image. Finally, the measurements for each eggshell were averaged and reported.

X-ray computed microtomography (GE Nanotom 180S, GE Sensing & Inspection Technologies GmbH, Wunstorf, German; voltage: 45 kV, cathode current: 350 μ A) was used for examination of the eggshells. Samples were scanned with a voxel size of 0.8 μ m. 3D image reconstruction, based on 1,200 2D images recorded during the scan, was performed using DatosX 2.0 software (GE

Sensing & Inspection Technologies GmbH, Wunstorf, German). In the next step, a 1.36×1.44 mm region of interest (ROI) was selected and subjected to analyses. Specific surface area of internal and external surfaces of the eggshells was determined using BoneJ plugin's "iso-surface" and "volume fraction" functions in Fiji software (NIH, Bethesda, MA, USA). For image visualization, Avizo 9 software (FEI, Hillsboro, Oregon, USA) was used. Measurements were performed for eggshell samples obtained from one egg from each replicate cage.

Measurements of eggshell mineral phase

The composition of the eggshell mineral phase was determined using ICP-OES spectrometry (iCAP Series 6500, Thermo Scientific, Waltham, MA, USA). Mineralization of the samples was conducted in a Microwave Digestion System (Berghof Speedwave, Eningen, Germany). The TraceCERT multi-element stock solution (#43843, Sigma-Aldrich, St. Louis, MO, USA) was used to prepare reference standards. The final results were expressed in mg or μ g per 1 g of dry eggshell sample.

The mean size of eggshell CaCO_3 crystals was measured using the XRD method. Analyses were performed using Empyrean X-ray diffractometer (PANalytical, Almelo, The Netherlands) in θ - 2θ geometry over a range from 10 to 80 deg with a step size of 0.01 deg and counting time 6 s per data point. Miller index (104) of CaCO_3 crystals (calcite) was taken for the calculation of eggshell calcium carbonate crystals size (Zhang et al., 2018). The peak position and FWHM (full-width at half-maximum) were calculated from the fits of the Voight function using Origin 9.0 software. Calculations of calcite crystals size were performed according to the Scherrer equation with the shape constant of 0.94 and apparatus broadening of 0.01 deg.

Statistical analysis

For all analyses, a replicate cage was considered as the experimental unit ($n = 12$ per group). The sample size was calculated for a two-tailed Student's t-test with an α of 0.05 and power at 0.8 (Festin, 2018). Available literature data show that for these assumptions sample size of $n = 12$ has a power of 80% to detect a change of 3.9%, 20% and 8.1% in eggshell Ca, P and Mg content, respectively, a change of 6.4% and 6.8% in the eggshell thickness and weight, respectively, a change of 22% in eggshell cracking force and stiffness (Ahmed et al., 2005; dos Santos et al., 2014; Park et al., 2018; Mori et al., 2020). The normal distribution of the datasets was checked using a Shapiro-Wilk test; the homogeneity of variances was assessed using Levene's test. Normally distributed variables were analyzed using a two-tailed Student's t-test or t-test with Welch's correction when datasets lacked equal variances. Non-parametric datasets were analyzed using Mann-Whitney U test. For all tests, a P-value < 0.05 was established as statistically significant. Effect size (ES) for all significant parametric comparisons was estimated with Cohen's d; Cohen's r was used for non-parametric

comparisons (Fritz et al., 2012). All statistical procedures were conducted using Statistica software (v. 13.3, TIBCO Software Inc., USA).

Results

All parameters related to egg quality, including the mathematical description of hen egg geometry were unchanged following Gln supplementation to laying hens for 30 weeks (Table 2). However, dietary supplementation of Gln resulted in an increase in the eggshell thickness (by 12.5%, ES: 0.96) and porosity (by 27.2%, ES: 1.30) (Table 3), and influenced the external specific surfaces of the eggshell (Figure 1). In Gln-supplemented hens, the external specific surface was flatter (ES: 0.56), while the internal specific surface was more heteroge-

neous (ES: 0.57), compared to control eggshells (Table 3).

While there was no effect of Gln supplementation on eggshell cracking force, work needed to crack the eggshell, as well as percentage deformation upon cracking, increased by 23.6% (ES: 1.05) and 12.9% (ES: 0.55), respectively, and eggshell stiffness and Young's modulus decreased by 12.6% (ES: 0.87) and 20.5% (ES: 0.41), respectively, following Glu supplementation (Table 3).

Dietary supplementation of Gln to laying hens increased the Ca content of the eggshell by 5.5% (ES: 4.94) and decreased the Cu, K, S, and Zn content (by 27.9%, ES: 2.12; by 5.5%, ES: 1.09; by 16.0%, ES: 1.48; and by 35.3%, ES: 2.40, respectively). No other changes were observed with regard to the mineral content of the eggshells. The size of CaCO_3 crystals forming eggshell also did not differ between groups (Table 4).

Table 2. The effect of dietary L-glutamine (Gln) supplementation (1.0%) on the quality (egg geometry, albumen and yolk indices) of eggs from Bovans Brown laying hens at the end of a 30-week supplementation period (31–60 weeks of age)

Item	Control	Gln	P-value
Egg weight (g)	60.8±0.9	62.6±0.5	0.104
Short axis (mm)	43.8±0.2	43.9±0.1	0.733
Long axis (mm)	56.7±0.5	57.2±0.4	0.140
Geometric mean diameter (mm)	47.7±0.3	47.9±0.2	0.508
Volume (cm ³)	57.5±1.0	58.2±0.5	0.558
Surface (cm ²)	71.8±0.8	72.4±0.5	0.508
Shape index	1.29±0.01	1.30±0.01	0.571
Specific gravity (g/cm ³)	1.081±0.002	1.085±0.001	0.185
Color of eggshell	25.4±0.4	25.3±0.5	0.900
Shell fraction (%)	12.7±0.5	12.9±0.2	0.691
Yolk fraction (%)	27.0±0.6	27.4±0.6	0.646
Albumen fraction (%)	60.3±0.6	59.7±0.7	0.519

Data are expressed as the means±SEM (n = 12). Significance was established using a two-tailed Student's t-test (normally distributed data), t-test with Welch's correction (normally distributed data with unequal variances) or the Mann–Whitney test (for pairwise comparisons with at least one non-normally distributed dataset).

Table 3. The effect of dietary L-glutamine (Gln) supplementation (1.0%) on eggshell quality of eggs from Bovans Brown laying hens at the end of a 30-week supplementation period (31–60 weeks of age)

Item	Control	Gln	P-value
Eggshell weight (g)	7.79±0.31	8.17±0.12	0.088
Eggshell thickness (mm)	0.428±0.007	0.460±0.005	0.001
Porosity (mm ²)	125±9	159±6	0.003
Internal side specific surface	2.26±0.03	2.64±0.05	<0.001
External side specific surface	1.13±0.01	1.10±0.01	<0.001
Cracking force (N)	49.5±2.0	51.5±1.6	0.443
Work to cracking (mJ)	11.0±0.7	13.6±0.6	0.013
Stiffness (N/mm)	119±6	104±3	0.036
Deformation (%)	1.56±0.05	1.75±0.05	0.007
Firmness (N/mm)	56.8±2.3	52.2±1.7	0.124
Young's modulus (GPa)	12.2±0.8	9.8±0.3	0.008
Fracture toughness (N/mm ^{3/2})	329±16	318±10	0.575

Data are expressed as the means±SEM (n = 12). Significance was established using a two-tailed Student's t-test (normally distributed data), t-test with Welch's correction (normally distributed data with unequal variances) or the Mann–Whitney test (for pairwise comparisons with at least one non-normally distributed dataset).

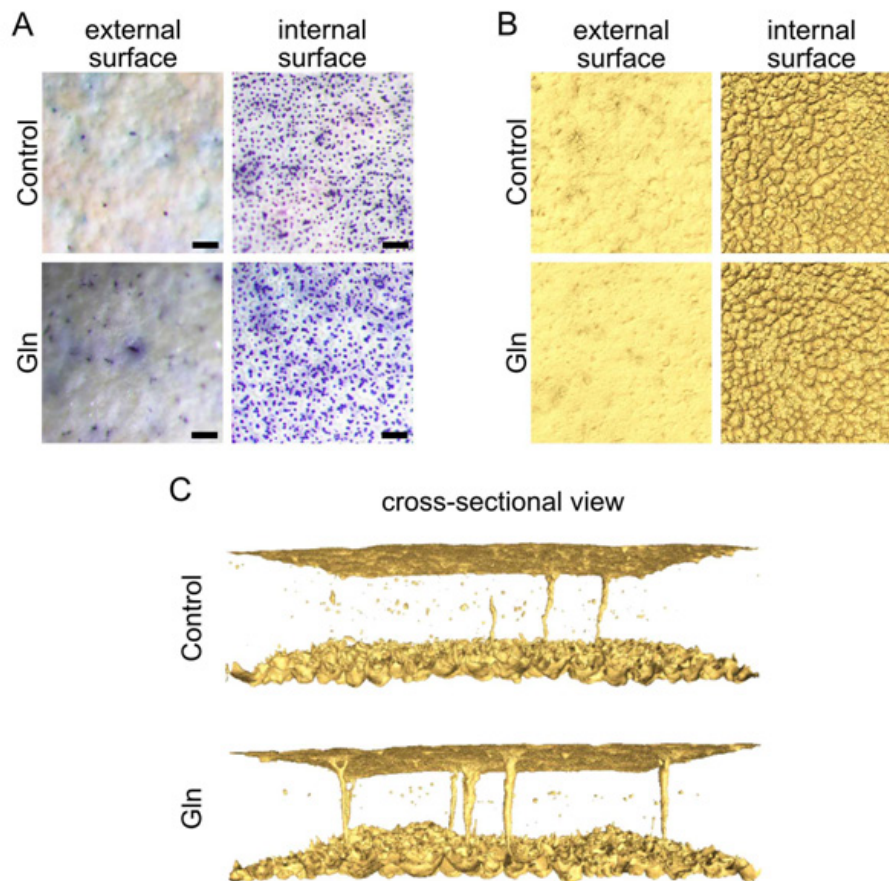


Figure 1. Microscope photographs and 3D reconstruction images of μ CT scans of the external and internal surfaces of eggshells from control (non-supplemented) and experimental (Gln, supplemented with L-glutamine at the dose of 1.0%) hens at the end of a 30-week supplementation period. (A) The internal surface of the eggshell was stained with methylene blue to visualize the pores on both sides of the eggshell. Scale bars: 250 μ m. (B) 3D reconstruction image of internal and external surface of the eggshell. (C) 3D reconstruction image of vertical ultrastructure of the eggshell. Microscopic images show changes in pore distribution after Gln supplementation, while 3D reconstruction images illustrate that Gln supplementation influences specific surfaces of the internal and external egg surfaces

Table 4. The effect of dietary L-glutamine (Gln) supplementation (1.0%) on eggshell mineral content of eggs and CaCO_3 crystal size from Bovans Brown laying hens at the end of a 30-week supplementation period (31–60 weeks of age)

Item	Control	Gln	P-value
Ca (mg/g)	330 $\pm$ 1	347 $\pm$ 1	<0.001
Co (μ g/g)	0.053 $\pm$ 0.003	0.055 $\pm$ 0.003	0.722
Cr (μ g/g)	0.732 $\pm$ 0.012	0.717 $\pm$ 0.006	0.269
Cu (μ g/g)	0.836 $\pm$ 0.037	0.603 $\pm$ 0.022	<0.001
Fe (μ g/g)	5.81 $\pm$ 0.04	5.85 $\pm$ 0.03	0.430
K (μ g/g)	738 $\pm$ 12	696 $\pm$ 8	0.011
Mg (mg/g)	3.37 $\pm$ 0.05	3.36 $\pm$ 0.06	0.902
Na (μ g/g)	1309 $\pm$ 7	1330 $\pm$ 24	0.424
P (mg/g)	1.36 $\pm$ 0.02	1.40 $\pm$ 0.02	0.173
S (μ g/g)	1203 $\pm$ 42	1010 $\pm$ 31	0.001
Se (μ g/g)	0.481 $\pm$ 0.046	0.505 $\pm$ 0.030	0.665
Si (μ g/g)	54.6 $\pm$ 0.2	54.3 $\pm$ 0.1	0.234
Zn (μ g/g)	0.61 $\pm$ 0.02	0.39 $\pm$ 0.03	<0.001
CaCO_3 crystal size (μ m)	57.8 $\pm$ 0.9	58.2 $\pm$ 1.0	0.408

Data are expressed as the means $\pm$ SEM (n = 12). Significance was established using a two-tailed Student's t-test (normally distributed data), t-test with Welch's correction (normally distributed data with unequal variances) or the Mann–Whitney test (for pairwise comparisons with at least one non-normally distributed dataset).

Discussion

The quality of eggshells is primarily influenced by the genetic origin of the laying hens, as well as their laying rate, health status, housing conditions, age, and most importantly their nutrition (Hamilton, 1982; Świątkiewicz et al., 2018). Supplementation with 1.0% Gln for 30 weeks did not have any detrimental effects on egg quality, including egg geometry, at the end of the supplementation period. However, the analysis of the quality of eggshells showed beneficial effects of Gln on the eggshell thickness and some mechanical parameters. Eggshell quality relates primarily to shell strength and resistance against cracking or breaking (De Ketelaere et al., 2002). The most common direct method for the measurement of eggshell strength is mechanical testing, which determines the precise force required to cause eggshell cracking (De Ketelaere et al., 2002; Solomon, 2010). Both cracking force and fracture toughness, which characterizes the sensitivity of the eggshell, as a biologically brittle material, to the occurrence of cracks, were the same in both groups irrespective of Gln supplementation. However, analysis of the other results obtained from the mechanical testing in our study showed an increase in deformation and decrease in stiffness of the eggshell following Gln supplementation to laying hens. Additionally, an increase in the amount of work required to crack the eggshell was observed in eggs obtained from Gln-supplemented hens. This means that at the same force, eggshells from Gln-supplemented hens were found to deform more than those from control hens, without cracking, and more work was required to crack the eggshell or, in other words, more energy was absorbed before cracking than that observed in the control eggs.

Both mechanical endurance of an eggshell and quality of its external surface are important from the point of view of sanitary considerations, since eggshell protects the internal parts of the egg against microorganisms from the external environment. The computer tomography of the eggshell showed that the external surface of the eggshell was smoother in eggs from Gln-supplemented hens, which could be considered worse for the adhesion of any microorganisms. Furthermore, a smoother eggshell surface ensures less soiling, which can also limit bacterial translocation in the case of microcracks.

Gln supplementation to the hens in the current study resulted in increased porosity of their eggshells. Pores ensure the maintenance of humidity and allow for the exchange of gases and thus the respiration of the embryo. Water loss from the egg, as well as gas conductance are dependent on the geometry of the pore, including total pore area and length (Ar et al., 1974; Mortola, 2009). However, the ratio of functional pore area to shell thickness, which determines the conductance of all gases across the eggshell, was not calculated in the present study and should be further investigated.

Given that the eggshell contains about 94% calcium, the most important factor which influences the mineral-

ization of the eggshell is adequate calcium supply (Goran et al., 2010; Athanasiadou et al., 2018). The two main sources of calcium are the diet and calcium turnover in bones of the laying hens. The determination of bone quality in our hens showed that Gln supplementation improves bone metabolism (Tomaszewska et al., 2020). This beneficial effect of Gln on bone turnover has been attributed to improved feed utilization, which can also result in better calcification of the eggshell, as was observed in our Gln-supplemented laying hens. Gln probably improved the mobilization of calcium from the bone and intensified the absorption of dietary calcium from the intestines, which resulted in an increase in the calcium content of the eggshells from our hens. Calcium-carbonate crystals (calcine) are deposited in the mammillary layer of the eggshell, in the cone-shaped mammillary knobs. It has been suggested that the larger the mammillary knobs, the higher the resistance of the eggshell to breaking/cracking (Radwan et al., 2010). Computer tomography analysis showed that the internal surface, containing the mammillary knobs, was significantly larger/thicker in the eggshells from Gln-supplemented hens compared to the control hens. However, based on the results of the XRD analysis, which showed that the mean thickness of the lateral size of the calcine crystal units constituting the eggshell, about 58 μm , typical for ca. 60-week-old hens (Rodriguez-Navarro et al., 2002), did not differ between groups, we can assume that the palisade layer size was unaffected by Gln supplementation, which additionally supports the results of mechanical testing, where no differences in cracking force were observed. However, this should be verified in future studies.

Many of the minerals, like zinc, copper, manganese, and selenium, are essential elements that are required in small amounts to ensure eggshell hardness, and are needed for the normal development and growth of the embryo (Murray et al., 2000). In the eggshells from our Gln-supplemented hens, a reduction in copper, sulfur, zinc, potassium was observed. Sulphur is important for the immune function of the embryo, the condition of the skin and its products in developing birds. Copper is an element of the active centers of many enzymes and plays a role as a cofactor of many enzymatic reactions; it also participates in the formation of red blood cells and their proper functioning, and regulates general and bone metabolism. Zinc plays a similar role to copper. Potassium is required for the proper functioning of the nervous system and for basal cell membrane activity. Changes in the composition of these minerals which make up the eggshell should be further studied with regards to their influence on avian development.

Despite the undoubtedly positive results, the presented study cannot be considered as final, as the present study has some limitations – such as using a one-time point in laying cycle in which eggs were analyzed. Therefore, while the obtained results clearly showed novel aspects of 1% Gln supplementation to laying hens, i.e. changes in eggshell quality in the post-peak laying period, further

research is needed, in which different periods of supplementation should be taken into consideration.

References

- Ahmed A.M.H., Rodriguez-Navarro A.B., Vidal M.L., Gautron J., Garcia-Ruiz J.M., Nys Y. (2005). Changes in eggshell mechanical properties, crystallographic texture and in matrix proteins induced by moult in hens. *Br. Poult. Sci.*, 46: 268–279.
- Altuntas E., Sekeroglu A. (2008). Effect of egg shape index on mechanical properties of chicken eggs. *J. Food. Eng.*, 85: 606–612.
- Ar A., Paganelli C.V., Reeves R.B., Greene D.G., Rahn H. (1974). The avian egg: Water vapor conductance, shell thickness, and functional pore area source. *Condor*, 76: 153–158.
- Athanasiadou D., Jiang W., Goldbaum D., Saleem A., Basy K., Pacella M.S., Böhm C.F., Chromik R.R., Hincke M.T., Rodríguez-Navarro A.B., Vali H., Wolf S.E., Gray J.J., Buy K.H., McKee M.D. (2018). Nanostructure, osteopontin, and mechanical properties of calcitic avian eggshell. *Sci. Adv.*, 4: eaar3219.
- Bain M.M. (1990). Eggshell strength: A mechanical/ultrastructural evaluation. Ph.D. thesis, University of Glasgow, UK.
- Bartell S.M., Batal A.B. (2007). The effect of supplemental glutamine on growth performance, development of the gastrointestinal tract, and humoral immune response of broilers. *Poultry Sci.*, 86: 1940–1947.
- Burrin D.G., Stoll B. (2009). Metabolic fate and function of dietary glutamate in the gut. *Am. J. Clin. Nutr.*, 90: 850S–856S.
- De Ketelaere B., Govaerts T., Coucke P., Dewil E., Visscher J., Decuyper E., De Baerdemaeker J. (2002). Measuring the eggshell strength of 6 different genetic strains of laying hens: Techniques and comparisons. *Br. Poult. Sci.*, 43: 238–244.
- Dos Santos F., Brandão M., Da Silva C., Machado L.D.S., Soares M., Barreto M., Nascimento E.D., Pereira V. (2014). Eggshell apex abnormalities in a free-range hen farm with *Mycoplasma synoviae* and infectious bronchitis virus in Rio de Janeiro state, Brazil. *Braz. J. Poult. Sci.*, 16: 101–103.
- Drabik K., Batkowska J., Vasiukov K., Putam A. (2020). The impact of eggshell colour on the quality of table and hatching eggs derived from Japanese quail. *Animals*, 10: 264.
- EFSA, European Food Safety Authority (2020). Safety and efficacy of L-glutamine produced using *Corynebacterium glutamicum* NITE BP-02524 for all animal species. *EFSA J.*, 18: 6075.
- EU, European Union, Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. *Off. J. Eur. Union*, 54: 1–130.
- Festin M.F.W. (2018). On determining sample size in experiments involving laboratory animals. *Lab. Anim.*, 52: 341–350.
- Fritz C.O., Morris P.E., Richler J.J. (2012). Effect size estimates: Current use, calculations, and interpretation. *J. Exp. Psychol.*, 141: 2–18.
- Gholipour V., Camani M., Aghdam Shahryar H., Sadeghi A.A., Aminafshar M. (2017). Effects of dietary L-glutamine supplemented on performance, egg quality, fertility and some blood biochemical parameters in guinea fowls (*Numida meleagris*). *Kafkas Univ. Vet. Fak. Derg.*, 23: 903–910.
- Gholipour V., Camani M., Aghdam Shahryar H., Sadeghi A.A., Aminafshar M. (2019). Effects of dietary L-glutamine supplement on performance, characteristics of the carcase and intestinal morphometry in guinea fowl chickens (*Numida meleagris*). *Ital. J. Anim. Sci.*, 18: 513–521.
- Goran G.V., Crivineanu V., Tudoreanu L., Udrea D. (2010). Dynamics of some mineral elements in hen eggs. *Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca Vet. Med.*, 67: 88–95.
- Hamilton R.M.G. (1982). Methods and factors that affect the measurement of egg shell quality. *Poultry Sci.*, 61: 2022–2039.
- Jazideh F., Farhoomand P., Daneshyar M., Najafi G. (2014). The effects of dietary glutamine supplementation on growth performance and intestinal morphology of broiler chickens reared under hot conditions. *Turk. J. Vet. Anim. Sci.*, 38: 264–270.
- Kibala L., Rozempolska-Rucińska I., Kasperek K., Zięba G., Łukaszewicz M. (2015). Ultrasonic eggshell thickness measurement for selection of layers. *Poultry Sci.*, 94: 2360–2362.
- Luquetti B.C., Fernandez Alarcon M.F., Lunedo R., Borges Campos D.M., Furlan R.L., Macari M. (2016). Effects of glutamine on performance and intestinal mucosa morphometry of broiler chickens vaccinated against coccidiosis. *Sci. Agric.*, 73: 322–327.
- Mabe I., Rapp C., Bain M.M., Nys Y. (2003). Supplementation of a corn-soybean meal diet with manganese, copper, and zinc from organic or inorganic sources improves eggshell quality in aged laying hens. *Poultry Sci.*, 82: 1903–1913.
- Mohsenin N.N. (1986). Physical properties of plant and animal materials: structure, physical characteristics and mechanical properties. 2nd ed. Gordon and Breach, London.
- Mori H., Takaya M., Nishimura K., Goto T. (2020). Breed and feed affect amino acid contents of egg yolk and eggshell color in chickens. *Poultry Sci.*, 99: 172–178.
- Mortola J.P. (2009). Gas exchange in avian embryos and hatchlings. *Comp. Biochem. Physiol. A*, 153: 359–377.
- Murray R.K., Granner D.K., Mayes P.A., Rodwell V.W. (2000). *Harpur's Biochemistry*. 25th ed. McGraw-Hill Medical.
- Narushin V.G. (2005). Egg geometry calculation using the measurements of length and breadth. *Poultry Sci.*, 84: 482–484.
- Nys Y., Le Roy N. (2018). Calcium homeostasis and eggshell biomineralization in female chicken. In: *Vitamin D. Volume 1: Biochemistry, physiology and diagnostics*, Feldman D. (ed.). 4th Ed., Academic Press, pp. 361–382.
- Orczewska-Dudek S., Pietras M., Puchała M., Nowak J. (2020). *Camelina sativa* oil and camelina cake as sources of polyunsaturated fatty acids in the diets of laying hens: effect on hen performance, fatty acid profile of yolk lipids, and egg sensory quality. *Ann. Anim. Sci.*, 20: 1365–1377.
- Park J.A., Shon S.H. (2018). The influence of hen aging on eggshell ultrastructure and shell mineral components. *Korean J. Food Sci. Anim. Res.*, 38: 1080–1091.
- Radwan L.M., Fathi M.M., Galal A., Zein El-Dein A. (2010). Mechanical and ultrastructural properties of eggshell in two Egyptian native breeds of chicken. *Int. J. Poult. Sci.*, 9: 77–81.
- Rodríguez-Navarro A., Kalin O., Nys Y., Garcia-Ruiz J.M. (2002). Influence of the microstructure on the shell strength of eggs laid by hens of different ages. *Br. Poult. Sci.*, 43: 395–403.
- Roudybush T., Hoffman L., Rahn H. (1980). Conductance, pore geometry, and water loss of eggs of Cassin's Auklet. *Condor*, 82: 105–106.
- Sakamoto M.I., Murakami A.E., Silveira T.G.V., Fernandes J.I.M., De Oliveira C.A.L. (2006). Influence of glutamine and vitamin E on the performance and the immune responses of broiler chickens. *Braz. J. Poult. Sci.*, 8: 243–249.
- Smulikowska S., Rutkowski A. (2018). Editors. Recommended allowances and nutritive value of feedstuffs – poultry feeding standards (in Polish). 5th ed. The Kielanowski Institute of Animal Physiology and Nutrition, Jabłonna, Poland, PAS.
- Sokolowicz Z., Krawczyk J., Dykiel M. (2018). The effect of the type of alternative housing system, genotype and age of laying hens on egg quality. *Ann. Anim. Sci.*, 18: 541–555.
- Solomon S.E. (2010). The eggshell: strength, structure and function. *Br. Poult. Sci.*, 51(suppl1): 52–59.
- Soltan M.A. (2008). Effect of dietary organic acid supplementation on egg production, egg quality and some blood serum parameters in laying hens. *Int. J. Poult. Sci.*, 7: 613–621.
- Soltan M.A. (2009). Influence of dietary glutamine supplementation on growth performance, small intestine morphology, immune response and some blood parameters of broiler chickens. *Int. J. Poult. Sci.*, 8: 60–68.
- Świątkiewicz S., Arczewska-Włosek A., Krawczyk J., Szczurek W., Puchała M., Józefiak D. (2018). Effect of selected feed additives on egg performance and eggshell quality in laying hens fed a diet with standard or decreased calcium content. *Ann. Anim. Sci.*, 18: 167–183.
- Świątkiewicz S., Arczewska-Włosek A., Szczurek W., Calik J., Bederska-Łojewska D., Orczewska-Dudek S., Muszyński S., Tomaszewska E., Józefiak D. (2020). Algal oil as source of polyun-

- saturated fatty acids in laying hens nutrition: Effect on egg performance, egg quality indices and fatty acid composition of egg yolk lipids. *Ann. Anim. Sci.*, 20: 961–973.
- Tomaszewska E., Świątkiewicz S., Arczewska-Włosek A., Wojtysiak D., Dobrowolski P., Domaradzki P., Świetlicka I., Donaldson J., Hulaś-Stasiak M., Muszyński S. (2020). Alpha-ketoglutarate: An effective feed supplement in improving bone metabolism and muscle quality of laying hens: A preliminary study. *Animals*, 10: 2420.
- Tyler C. (1953). Studies on egg shells. II – A method for marking and counting pores. *J. Sci. Food Agric.*, 4: 266–272.
- Xiao D., Zeng L., Yao K., Kong X., Wu G., Yin Y. (2016). The glutamine-alpha-ketoglutarate (AKG) metabolism and its nutritional implications. *Amino Acids*, 48: 2067–2080.
- Xiao J.F., Zhang Y.N., Wu S.G., Zhang H.J., Yue H.Y., Qi G.H. (2014). Manganese supplementation enhances the synthesis of glycosaminoglycan in eggshell membrane: A strategy to improve eggshell quality in laying hens. *Poultry Sci.*, 93: 380–388.
- Xue G.D., Barekattain R., Wu S.B., Choct M., Swick R.A. (2018). Dietary L-glutamine supplementation improves growth performance, gut morphology, and serum biochemical indices of broiler chickens during necrotic enteritis challenge. *Poultry Sci.*, 97: 1334–1341.
- Zavarize K.C., Sartori J.R., Pezzato A.C., Garcia E.A., Cruz V.C. (2011). Glutamine in diet of laying hens submitted to heat stress and thermoneutrality. *Ci. Anim. Bras.*, 12: 400–406.
- Zhang Y., Li H., Zhang Y., Song F. (2018). Statistical optimization and batch studies on adsorption of phosphate using Al-eggshell. *Adsorp. Sci. Technol.*, 36: 026361741774079.

Received: 21 XII 2021

Accepted: 15 II 2022