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L-ascorbic acid supplementation at different stocking densities on the performance of laying hens

Abstract – The objective of this work was to evaluate the effect of L-ascorbic acid supplementation at different stocking densities on the performance, egg quality, tonic immobility, and hematological parameters of laying hens. For this, 160 Hy-line W-80 white laying hens, with 58 weeks of age, were subjected to four treatments with ten replicates, combining stocking density (560 or 336 cm^2 per hen) and L-ascorbic acid supplementation (0 or 150 mg kg^{-1} of feed). The data were analyzed in a 2×2 factorial arrangement, using a general linear model. The high stocking density of 336 cm^2 per hen resulted in: a lower final body weight; a lower egg production; a higher feed intake; a higher feed conversion ratio; and no effect on egg yield, weight, mass, and shell quality, as well as on tonic immobility, serum glucose, cholesterol, and alanine aminotransferase. The high stocking density, irrespective of supplementation, reduced the Haugh unit and increased respiratory rate, feather loss, and the concentrations of serum triglycerides, aspartate aminotransferase, and cortisol. L-ascorbic acid supplementation improved feed intake and egg yolk color. However, supplementation does not eliminate the negative effects of the high stocking density on the performance, egg characteristics, or physiological parameters of laying hens.

Index terms: ascorbic acid, hematology, laying hens, stocking density, welfare.

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Suplementação de ácido L-ascórbico em diferentes densidades de alojamento no desempenho de galinhas poedeiras

Resumo – O objetivo deste trabalho foi avaliar o efeito da suplementação de ácido L-ascórbico em diferentes densidades de alojamento no desempenho, na qualidade dos ovos, na imobilidade tônica e nos parâmetros hematológicos de galinhas poedeiras. Para tanto, 160 galinhas poedeiras brancas Hy-line W-80, com 58 semanas de idade, foram submetidas a quatro tratamentos com dez repetições, combinando densidade de estocagem (560 ou 336 cm^2 por galinha) e suplementação de ácido L-ascórbico (0 ou 150 mg kg^{-1} de ração). Os dados foram analisados em arranjo fatorial 2 × 2, tendo-se utilizado um modelo linear geral. A alta densidade de estocagem de 336 cm^2 por galinha resultou em: menor peso corporal final; menor produção de ovos; maior consumo de ração; maior taxa de conversão alimentar; e nenhum efeito sobre produção, peso, massa e qualidade da casca dos ovos, bem como sobre imobilidade tônica, glicose sérica, colesterol e alanina aminotransferase. A alta densidade populacional, independentemente da suplementação, reduziu a unidade Haugh e aumentou a frequência respiratória, a perda de penas, e as concentrações de triglicerídeos séricos, aspartato aminotransferase e cortisol. A suplementação com ácido L-ascórbico melhorou o consumo de ração e a coloração da

gema do ovo. No entanto, a suplementação não elimina os efeitos negativos da alta densidade populacional sobre o desempenho, as características dos ovos ou os parâmetros fisiológicos das galinhas poedeiras.

Termos para indexação: ácido ascórbico, hematologia, aves poedeiras, densidade populacional, bem-estar animal.

Introduction

The poultry industry is rapidly expanding globally, with egg production increasing by 150% over the last three decades (FAO, 2023). However, considering that the world population is projected to reach 10 billion by 2050, there is a pressing need to enhance the quantity and quality of animal-derived products in order to meet nutritional demands (Gil et al., 2024).

Although poultry products are essential sources of vital nutrients, the increase in the production of poultry meat and egg may negatively affect animal welfare (Cao et al., 2024). To assess the well-being of birds, it is important to recognize stress and how it impacts their performance and physiological and behavioral functions (Bilal et al., 2021).

Legislation and advocacy for animal welfare, such as Directive 1999/74/EC (Council of the European Union 1999), highlight the importance of an adequate space allowance for laying hens. Therefore, stocking density (SD), defined as the number of birds per unit of available floor space (European Comission, 2023), is considered a critical factor affecting poultry health and production.

A high SD, for example, may enhance farm profitability per area, but may also pose significant threats to animal welfare. Among these, heating stress stands out, being related to body weight loss, low feed intake, low egg production, low egg quality, poor eggshell traits, respiratory problems, poor meat quality, low reproductive rate, immune deficiency, and high mortality (Mangan & Siwek, 2024). In this context, L-ascorbic acid emerges as a potential solution (Büyükkılıç Beyzi et al., 2020), especially due to its antioxidant properties and positive effects on the performance, metabolism, product quality, and immune system of laying hens (Hieu et al., 2022). In poultry, L-ascorbic acid supplementation has been associated with body temperature regulation, immune bolstering, enhanced antioxidant activity, and healthy gut microbiota (Shojadoost et al., 2021).

The objective of this work was to evaluate the effect of L-ascorbic acid supplementation at different stocking densities on the performance, egg quality, tonic immobility, and hematological parameters of laying hens.

Materials and Methods

The animal use protocol of this research was reviewed and approved by the Institutional Animal Care and Use Committee of Erciyes University, under approval number 21/20. The study was carried out at the Agricultural Research and Application Center of the same university, in the municipality of Kayseri, Türkiye.

During an adaptation period of two weeks, 160 Hy-line W-80 laying hens, aged 58 weeks, were categorized according to body weight, egg production, and egg weight in order to minimize differences among the studied groups. The experiment encompassed two levels of SD (low and high, with 560 and 336 cm^2 of cage floor per hen, respectively) and two of dietary L-ascorbic acid supplementation (0 and 150 mg kg^{-1} of feed), combined into four treatments with ten replicates (cages) each. The treatments (T1 to T4) were: T1, low SD and no L-ascorbic acid supplementation; T2, low SD and 150 mg of L-ascorbic acid per kilogram of feed; T3, high SD and no L-ascorbic acid supplementation; and T4, high SD and 150 mg of L-ascorbic acid per kilogram of feed. For the low and high SDs, three and five hens were kept per cage, respectively, totaling 160 birds. The choice of 150 mg kg^{-1} of L-ascorbic acid supplementation was based on findings from a previous study that showed the beneficial effects of this dosage on poultry under stress conditions (Ajakaiye et al., 2010).

The hens were housed in a total of 40 conventional wire cages (42 cm length x 40 cm width x 46 cm height) with three floors. The feeders were partitioned to restrict feed consumption from neighboring cages.

During the experiment, the hens were reared under consistent and semi-regulated environmental settings, which included monitored temperatures and humidity, automated lighting system, and adjustable airflow through windows and roof holes. The lighting schedule was of 16 hours, starting at 5:00 a.m. and ending at 9:00 p.m.

Diets and water were available ad libitum. The basal diet, formulated to meet the nutritional requirements of laying hens according to the breed's guide (Hy-Line International, 2024), consisted of corn, soybean, sunflower, dried distillers' grains with solubles, vegetable oil, vitamin and mineral premix, amino acids, and mycotoxin binder. The chemical content of the basal diet was 89.28% dry matter, 18.10% crude protein, 4.70% crude fat, 4.98% crude fiber, 12.24% crude ash, 3.80% calcium, 0.35% available phosphorus, 0.40% methionine, 0.80% lysine, and 2,780 kcal metabolizable energy per kilogram of feed.

The body weight of the evaluated 160 laying hens was measured at the beginning and at the end of the study. Feed intake was calculated every 14 days through the difference between the provided and remaining feed. Average daily feed intake was obtained by dividing feed intake during 14 days by the number of animals. The feed conversion ratio (FCR) was determined as the ratio of feed intake to egg mass per period. The egg mass value was calculated by multiplying egg weight and egg production percentage in the relevant period.

Egg production was monitored daily for each cage. Egg yield, also for each cage, was calculated biweekly as a percentage, obtained by dividing the total number of eggs produced in n days by n days and then by the number of hens in the cage. To determine the traits of egg internal quality and shell quality, every two weeks, 60 eggs of each treatment group (240 eggs in total) were randomly sampled during the last three days of each period. The traits of egg weight and egg internal quality, including albumin height, Haugh unit, and Roche yolk color scale (RCS), were evaluated using the EggAnalyzer equipment (Orka Food Technology LLC, South West Bountiful, UT, USA). Egg yolk color as CIE-Lab color parameters (L*, brightness; a*, redness; and b*, yellowness) was evaluated using the CR-400 chroma meter (Konica Minolta Inc., Tokyo, Japan). Each measurement was repeated three times at different points, and the calculated means were used in the data analysis.

The shells of the 60 eggs of each group (240 eggs in total) were broken, washed with tap water, and dried, at 75°C, for 24 hours in an oven. After drying, eggshell weight was determined using an analytical balance, with an accuracy of ±0.1 g. Eggshell percentage was calculated by dividing eggshell weight by egg weight and then by multiplying the obtained value by 100. Additionally, the thickness of the eggshells (sharp, blunt, and middle parts) was measured with a digital micrometer (±0.01 mm), and arithmetic means were obtained.

Feed nutritional analyses, including dry matter, crude protein, crude ash, and crude fat, were carried out according to Association of Official Analytical Chemists (AOAC) (Latimer Jr., 2023). The used feed additives were supplied by Farmavet International (Tuzla, Istanbul, Türkiye).

To assess the tonic immobility of the 160 hens, 1 individual from each subgroup was selected. The chosen hens were placed in a quiet and stable environment to evaluate their ability to keep still before exhibiting signs of restlessness. A stopwatch was used to measure the time each hen remained in a state of immobility during a period ranging from 10 to 600 s, equivalent to 10 min (Campo & Dávila, 2002). Additionally, using a stopwatch, the respiration rate of one hen from each subgroup was measured in a 2 min interval.

Feather loss scores were determined for each of the 160 hens at the end of the trial, following the AssureWel system (AssureWel, 2013). In this method, the dorsal/ventral area and head/neck area of the hens are evaluated visually for lesions, without any type of handling.

On the last day of the trial, in the sixteenth week, 10 mL blood samples were collected from the wing vein of a randomly selected laying hen from each replicate (cage), i.e., ten hens from each treatment group, totaling 40 samples overall. This was done to ensure that each group was equally represented. The blood samples were centrifuged at 2,500 rpm for 5 min, and the serum was separated and stored in Eppendorf tubes, at -80°C, until the day of the analyses for serum glucose, total protein, triglyceride cholesterol, high-density lipoprotein, aspartate aminotransferase, and alanine aminotransferase. Commercial kits and an auto-analyzer were used.

For blood leukocyte cell counting, a total of 40 blood samples were collected from 10 hens from each subgroup, also in the sixteenth week. The blood samples were smeared onto glass slides, fixed using methyl alcohol, and stained with Wright stain. Using a microscope at 100× magnification, heterophils, lymphocytes, basophils, eosinophils, and monocytes

were counted, and the heterophil:lymphocyte ratio was calculated according to Gross & Siegel (1986).

The data were checked for assumptions of normality of residuals and homogeneity of variances by the tests of Shapiro-Wilk ($p > 0.05$) and Levene ($p > 0.05$); both assumptions were met. The independence of errors was ensured by the experimental design, as replicate cages were treated as independent experimental units. Therefore, no additional test of independence was required. The data related to SD, L-ascorbic acid, and their interactions were analyzed using a two-way analysis of variance in the SPSS statistics software, version 25 (IBM, Armonk, NY, USA). Duncan's test was employed to identify differences between means ($\alpha = 0.05$). The obtained results were reported as mean and standard error. Feed and egg trait data were collected biweekly, being summarized and analyzed separately at four- and eight-week intervals.

Results and Discussion

L-ascorbic acid supplementation and SDs affected body weight, feed intake, and the FCR in laying hens (Table 1). The high SD reduced final body weight and the weight change ratio ($p < 0.05$), indicating the negative effect of crowding stress due to a limited space. This observation aligns with the studies of Weimer et al. (2019) and Wang et al. (2020), who reported a decreasing final body weight under high SD conditions. However, irrespective of the SD, the concentrations of L-ascorbic acid supplementation did not affect body weight or the weight change ratio of laying hens in the present work.

The higher SD led to an increased feed intake among laying hens across the four-week, eight-week, and overall interval periods ($p < 0.05$), possibly as a response to the increased stress and competition for resources. However, Benyi et al. (2006) concluded that a lower SD (1,100 cm^2 per hen) increased final body weight and feed intake, whereas a high SD (550 cm^2 per hen) did not affect the FCR values. This latter result contrasts with that of Kang et al. (2016), who associated a higher SD with a lower feed intake in laying hens. In the present study, SD had a significant effect on the FCR values throughout all interval periods ($p < 0.05$), a pattern also reported by Kahraman et al. (2020) and Kaya et al. (2021). This suggests that laying hens under

Table 1. Results of the mean (standard error) and p-value obtained for the body weight, feed intake, and feed conversion ratio of Hy-line W-80 laying hens due to the main effects of L-ascorbic acid (AA) supplementation, stocking density (SD), and their interactions[1].

Variable	SD		AA[2]		Treatment (interactions)[3]				p-value		
	Low	High	Not suppl.	Supplemented	T1	T2	T3	T4	SD	AA	SD*AA
Body weight (g)											
Initial weight	1,482.30 (37.44)	1,449.35 (40.56)	1,397.70 (28.68)	1,433.95 (51.52)	1,434.40 (55.52)	1,361.00 (55.52)	1,530.20 (55.52)	1,337.70 (55.52)	0.220	0.518	0.291
Final weight	1,576.00 (37.15)a	1,408.35 (40.10)b	1,467.50 (30.68)	1,516.85 (52.18)	1,521.90 (54.69)	1,413.10 (54.69)	1,630.10 (54.69)	1,403.60 (54.69)	0.004	0.373	0.289
Body weight change (%)	6.42 (0.49)a	4.51 (0.43)b	4.99 (0.41)	5.95 (0.58)	5.74 (0.56)	3.71 (0.56)	6.27 (0.56)	4.86 (0.56)	0.004	0.146	0.585
Interval (weeks)					Feed intake (g)						
Four	98.06 (0.32)b	115.34 (0.54)a	106.25 (1.96)	107.15 (2.09)	97.93 (0.62)	98.20 (0.62)	114.58 (0.62)	116.10 (0.62)	0.000	0.155	0.320
Eight	104.19 (0.99)b	114.46 (0.72)a	108.09 (1.62)b	110.56 (1.21)a	101.98 (1.14)	106.40 (1.14)	114.20 (1.14)	114.73 (1.14)	0.000	0.037	0.096
Overall	101.13 (0.47)b	114.90 (0.48)a	107.17 (1.70)b	108.86 (1.57)a	99.95 (0.61)	102.30 (0.61)	114.39 (0.61)	115.41 (0.61)	0.000	0.009	0.288
Interval (week)					Feed conversion ratio (g feed per gram of egg mass)						
Four	1.62 (0.02)b	1.83 (0.02)a	1.74 (0.02)	1.71 (0.02)	1.64 (0.03)	1.59 (0.03)	1.84 (0.03)	1.83 (0.03)	0.000	0.436	0.488
Eight	1.64 (0.03)b	1.83 (0.03)a	1.73 (0.03)	1.74 (0.03)	1.62 (0.05)	1.67 (0.05)	1.85 (0.05)	1.81 (0.05)	0.000	0.965	0.329
Overall	1.63 (0.03)b	1.83 (0.03)a	1.73 (0.03)	1.72 (0.03)	1.63 (0.04)	1.63 (0.04)	1.84 (0.04)	1.82 (0.04)	0.000	0.754	0.838

[1]Means followed by different letters in the same line differ significantly by Duncan's test ($p < 0.05$). [2]No suppl., no supplementation; and Suppl.; supplementation. [3]T1, low SD and no AA supplementation; T2, low SD and 150 mg of AA per kilogram of feed; T3, high SD and no AA supplementation; and T4, high SD and 150 mg of AA per kilogram of feed.

a high SD experience stress and competition, which reduces their productive efficiency.

As to the supplementation with L-ascorbic acid, Büyükkılıç Beyzi et al. (2020) pointed out that it is a common practice to protect laying hens against heat stress. Under these conditions, endogenous L-ascorbic acid is insufficient to meet the requirements of the birds, activating catecholamine hormones, which rapidly deplete the body's L-ascorbic acid reserves (Puron et al., 1994). In this line, Abidin & Khatoon (2013) concluded that L-ascorbic acid may help poultry in situations of poor immunity, low feed intake, oxidative stress, increased body temperature, and mortality. However, in the present study, irrespective of SD, L-ascorbic acid supplementation had no effect on body weight and the FCR, despite increasing feed intake during the eight-week and overall interval periods ($p < 0.05$). These results support the findings of Seven (2008) and Saiz del Barrio et al. (2020) regarding body weight and feed intake. Abudabos et al. (2018) found that L-ascorbic acid supplementation significantly improved feed intake, while also increasing body weight gain. However, Torki et al. (2014) did not report any effect of L-ascorbic acid supplementation (250 mg kg^{-1}) on the FCR for laying hens.

The treatments with L-ascorbic acid supplementation affected egg weight and egg mass, but not egg production (Table 2). Contrastingly, Seven et al. (2008) and Torki et al. (2014) concluded there was no effect of L-ascorbic acid supplementation on egg mass. However, Skřivan et al. (2013) also observed that L-ascorbic acid supplementation (0 or 200 mg kg^{-1}) did not impact egg production or feed consumption.

Egg weight, mass, and production were affected by the two SDs. Egg weight and mass were higher at the low SD in the four-week interval period ($p < 0.05$), an effect that decreased at the eight-week and overall interval periods. These findings align with those of Benyi et al. (2006), who reported an increased egg production under a low SD. Erensoy et al. (2021) also verified that egg production increased at a low SD, decreasing at a high and medium SD. Likewise, Weimer et al. (2019) found that a high SD reduced egg production. In the present study, the decreased egg production at the high SD, despite a slight increase in feed intake, may be attributed to the competition among hens to obtain adequate feed and calcium intakes under crowded conditions.

Table 2. Results of the mean (standard error) and p-value obtained for the egg yield, egg weight, and egg mass of Hy-line W-80 laying hens due to the main effects of L-ascorbic acid supplementation (AA), stocking density (SD), and their interactions[1].

Interval (week)	SD		AA[2]		Treatment (interactions)[3]				p-value		
	Low	High	No suppl.	Suppl.	T1	T2	T3	T4	SD	AA	SD*AA
					Egg yield (%)						
Four	93.99 (0.51)	91.90 (1.07)	92.26 (1.02)	93.63 (0.66)	93.27 (1.20)	94.70 (1.20)	91.24 (1.20)	92.55 (1.20)	0.090	0.260	0.962
Eight	96.49 (0.600	95.09 (1.36)	95.50 (1.11)	96.08 (0.07)	96.67 (1.40)	96.31 (1.40)	94.33 (1.40)	95.86 (1.40)	0.326	0.678	0.504
Overall	95.24 (0.28)	93.50 (1.21)	93.88 (1.03)	94.86 (0.73)	94.97 (1.27)	95.51 (1.27)	92.78 (1.27)	94.21 (1.27)	0.177	0.444	0.728
					Egg weight (g)						
Four	65.59 (0.53)b	67.04 (0.36)a	65.78 (0.46)	66.85 (0.47)	64.88 (0.63)	66.30 (0.63)	66.68 (0.63)	67.40 (0.63)	0.028	0.099	0.582
Eight	65.89(0.46)	65.25 (0.59)	65.22 (0.59)	65.92 (0.46)	65.74 (0.76)	66.04 (0.76)	64.70 (0.76)	65.80 (0.76)	0.405	0.363	0.596
Overall	65.74 (0.37)	66.14 (0.39)	65.50 (0.40)	66.39 (0.34)	65.31 (0.53)	66.17 (0.53)	65.69 (0.53)	66.60 (0.53)	0.457	0.106	0.959
					Egg mass (g)						
Four	61.00 (0.54)b	63.47 (0.90)a	61.53 (0.90)	62.93 (0.63)	59.94 (1.05)	62.05 (1.05)	63.13 (1.05)	63.81 (1.05)	0.024	0.191	0.499
Eight	63.84 (0.50)	63.31 (1.06)	62.97 (0.93)	64.18 (0.71)	63.32 (1.20)	64.36 (1.20)	62.61 (1.20)	64.00 (1.20)	0.658	0.316	0.884
Overall	62.42 (0.43)	63.39 (0.94)	62.25 (0.74)	63.56 (0.74)	61.63 (1.04)	63.21 (1.04)	62.87 (1.04)	63.91 (1.04)	0.357	0.218	0.797

[1]Means followed by different letters in the same line differ significantly by Duncan's test ($p < 0.05$). [2]No suppl., no supplementation; and Suppl.; supplementation. [3]T1, low SD and no AA supplementation; T2, low SD and 150 mg of AA per kilogram of feed; T3, high SD and no AA supplementation; and T4, high SD and 150 mg of AA per kilogram of feed.

The effects of the treatments on albumin height, Haugh unit, eggshell weight, eggshell ratio, and eggshell thickness varied (Table 3). Irrespective of L-ascorbic acid supplementation, the high SD reduced albumin height and Haugh unit values during the four-week and overall interval periods ($p < 0.05$). For eggshell weight, no treatment effects were observed. As to the eggshell ratio, although it was not significantly affected by SD, it increased due to L-ascorbic acid supplementation ($p < 0.05$), decreasing at the high SD with supplementation at the eight-week interval period ($p < 0.05$). For eggshell thickness, the obtained values were higher both under the high SD ($p < 0.05$) and supplemented ($p < 0.05$) treatments in the four-week interval period.

In their study, Geng et al. (2020) concluded that SD values of 5, 6, 7, and 8 hens per square meter did not affect egg quality traits. Likewise, Son et al. (2020) found that SD in conventional cage systems (750 and 500 cm^2 per bird) did not have any significant effects on egg quality traits. In this line, Altan et al. (2002) reported that the SDs of 640 and 480 cm^2 per hen did not significantly affect egg production and egg weight, although, at 384 cm^2 per hen, Haugh unit and egg production were affected, but not egg weight, percentage, thickness, and ratio. Similarly, Mirfendereski & Jahanian (2015) reported that 5 to 7 laying hens per 1,800 cm^2 cage did not affect eggshell weight, thickness, or ratio.

Erensoy et al. (2021) concluded that laying hens under a low and medium SD produced more eggs than those at a high SD (1,104.5, 736.3, and 552.3 cm^2 of cage floor space per hen, respectively). Asghar Saki et al. (2012) observed that the cage densities of 2,000, 1,000, 667, and 500 cm^2, with four hens each, resulted in a significantly lower eggshell weight compared with that of the control group, with one hen per cage, whereas egg quality and egg production increased at 1,000 cm^2 with two hens.

Under heat stress conditions, Büyükkılıç Beyzi et al. (2020) found that, the addition of 250 mg kg^{-1} L-ascorbic acid did not affect egg quality traits in

Table 3. Results of the mean (standard error) and p-value of egg album height, Haugh unit, eggshell weight, eggshell ratio, and eggshell thickness of Hy-line W-80 laying hens due to the main effects of L-ascorbic acid (AA) supplementation, stocking density (SD), and their interactions[1].

Interval (weeks)	SD		AA[2]		Treatment (interactions)[3]				p-value		
	Low	High	Not suppl.	Suppl.	T1	T2	T3	T4	SD	AA	SD*AA
					Egg albumen height (mm)						
Four	4.79 (0.09)a	4.50 (0.10)b	4.68 (0.08)	4.60 (0.11)	4.70 (0.13)ab	4.87 (0.13)a	4.67 (0.13)b	4.32 (0.13)c	0.028	0.502	0.045
Eight	5.36 (0.25)	5.16 (0.25)	5.22 (0.25)	5.31 (0.25)	5.40 (0.36)	5.33 (0.36)	5.04 (0.36)	5.28 (0.36)	0.578	0.807	0.672
Overall	5.07 (0.13)	4.83 (0.14)	4.95 (0.14)	4.95 (0.14)	5.05 (0.20)	5.10 (0.20)	4.86 (0.20)	4.80 (0.20)	0.218	0.998	0.782
					Haugh unit						
Four	62.14 (1.00)a	58.21 (1.27)b	61.03 (1.08)	59.31 (1.34)	61.22 (1.53)ab	63.06 (1.53)a	60.85 (1.53)ab	55.57 (1.53)b	0.015	0.269	0.026
Eight	68.10 (2.42)	62.78 (1.03)	65.91 (0.89)	64.98 (2.62)	68.17 (2.70)	68.03 (2.70)	63.64 (2.70)	61.92 (2.70)	0.056	0.732	0.770
Overall	65.12 (1.42)a	60.49 (0.85)b	63.47 (0.59)	62.14 (1.71)	64.70 (1.65)	65.55 (1.65)	62.25 (1.65)	58.74 (1.65)	0.008	0.427	0.196
					Eggshell weight (g)						
Four	6.05 (0.05)	6.00 (0.04)	6.08 (0.04)	5.98 (0.05)	6.07 (0.06)	6.04 (0.06)	6.09 (0.06)	5.91 (0.06)	0.391	0.107	0.227
Eight	6.25 (0.05)	6.25 (0.06)	6.24 (0.05)	6.26 (0.03)	6.22 (0.08)	6.27 (0.08)	6.26 (0.08)	6.24 (0.08)	0.958	0.774	0.639
Overall	6.15 (0.04)	6.12 (0.03)	6.15 (0.03)	6.12 (0.04)	6.14 (0.05)	6.16 (0.05)	6.17 (0.05)	6.08 (0.05)	0.641	0.461	0.288
					Eggshell ratio (%)						
Four	11.00 (0.13)	11.31 (0.11)	10.97 (0.10)b	11.33 (0.13)a	10.89 (0.16)	11.12 (0.16)	11.06 (0.16)	11.55 (0.16)	0.074	0.036	0.430
Eight	10.64 (0.12)	10.58 (0.16)	10.57 (0.14)	10.66 (0.14)	10.66 (0.20)	10.63 (0.20)	10.48 (0.20)	10.69 (0.20)	0.770	0.657	0.549
Overall	10.82 (0.10)	10.94 (0.10)	10.77 (0.09)	11.00 (0.10)	10.77 (0.14)	10.87 (0.14)	10.77 (0.14)	11.12 (0.14)	0.383	0.111	0.357
					Eggshell thickness (µm)						
Four	34.32 (0.15)b	34.59 (0.18)a	34.00 (0.11)b	0.34.91 (0.15)a	0.34 (0.00)	0.35 (0.00)	0.34 (0.00)	0.35 (0.00)	0.036	0.000	0.696
Eight	33.59 (0.42)	33.38 (0.11)	33.35 (0.41)	33.63 (0.13)	0.34 (0.00)a	0.34 (0.00)a	0.33 (0.00)b	0.34 (0.00)a	0.516	0.388	0.000
Overall	33.96 (0.22)	33.97 (0.12)	33.67 (0.20)	34.27 (0.11)	0.34 (0.00)	0.34 (0.00)	0.34 (0.00)	0.34 (0.00)	0.696	0.057	0.696

[1]Means followed by different letters in the same line differ significantly by Duncan's test ($p < 0.05$). [2]No suppl., no supplementation; and Suppl.; supplementation. [3]T1, low SD and no AA supplementation; T2, low SD and 150 mg of AA per kilogram of feed; T3, high SD and no AA supplementation; and T4, high SD and 150 mg of AA per kilogram of feed.

laying hens. Similarly, Asli et al. (2007) noted that adding 200 mg kg^{-1} of L-ascorbic acid to the basal diet had no significant effect on eggshell thickness, shell percentage, and Haugh unit values.

Regarding yolk color measurement (Table 4), the high SD elevated the RCS values in the eight-week interval period ($p < 0.05$), with no effect during the four-week and overall interval periods. Moreover, L-ascorbic acid supplementation increased the RCS values in the four-week and overall interval periods ($p < 0.05$). However, there were no significant differences in the RCS values between the treatment groups. Wang et al. (2020) found that the RCS value decreased at a high SD compared to a low one (338 and 506 cm^2, respectively).

The supplementation of L-ascorbic acid led to a significant decrease in the L* values of the egg yolk during the four-week and overall interval periods ($p < 0.05$). However, there were no significant effects of the interactions between SD and L-ascorbic acid on these values. In terms of redness, during the eight-week interval period, the a* values in the egg yolk were higher under the high SD ($p < 0.05$), but lower with L-ascorbic acid supplementation, irrespective of SD, during the four-week and overall interval periods ($p < 0.05$). Additionally, regardless of SD, L-ascorbic acid supplementation decreased b* values during the four-week interval period ($p < 0.05$). This trend is in alignment with that reported by Şekeroğlu et al. (2010) regarding the lack of effect of SD variations on yolk color. Çiftçi et al. (2005), however, observed that L-ascorbic acid increased yolk color under heat stress conditions.

The effects of treatments on blood components, tonic immobility, respiratory rate, and feather loss in laying hens is shown in Table 5. High SD levels caused an increase in the concentrations of serum triglycerides, aspartate aminotransferase, and cortisol ($p < 0.05$), but did not significantly affect serum glucose, cholesterol, and alanine aminotransferase. These results reflect the physiological stress experienced by the hens under a high SD, indicating high cortisol levels. Von Eugen et al. (2019) found that a high SD led to higher corticosterone levels and anxiety in laying-hen chicks. In this line, Oluwagbenga et al. (2022) concluded that heat stress elevated cortisol concentrations in ducks.

Neither L-ascorbic acid supplementation nor SD influenced the serum components of the hens. Similarly, Guo et al. (2012) and Hanafy et al. (2022) highlighted that different SD did not affect the levels of

Table 4. Results of the mean (standard error) and p-value of the Roche yolk color scale, egg yolk brightness, egg yolk redness, and egg yolk yellowness of Hy-line W-80 laying hens due to the main effects of L-ascorbic acid (AA) supplementation, stocking density (SD), and their interactions[1].

Interval (weeks)	SD		AA[2]		Treatment (interactions)[3]				p-value		
	Low	High	No suppl.	Suppl.	T1	T2	T3	T4	SD	AA	SD*AA
Roche yolk color scale											
Four	5.41 (0.11)	5.29 (0.12)	4.95 (0.05)b	5.75 (0.09)a	4.99 (0.11)	5.82 (0.11)	4.91 (0.11)	5.68 (0.11)	0.295	0.000	0.793
Eight	6.03 (0.08)b	6.29 (0.05)a	6.16 (0.08)	6.15 (0.08)	6.03 (0.10)	6.03 (0.10)	6.29 (0.10)	6.28 (0.10)	0.016	0.966	0.973
Overall	5.72 (0.07)	5.79 (0.06)	5.56 (0.05)b	5.95 (0.05)a	5.51 (0.07)	5.92 (0.07)	5.60 (0.07)	5.98 (0.07)	0.266	0.000	0.783
Egg yolk brightness											
Four	59.78 (0.24)	60.06 (0.30)	60.63 (0.22)a	59.21 (0.23)b	60.31 (0.32)	59.25 (0.32)	60.95 (0.32)	59.16 (0.32)	0.391	0.000	0.258
Eight	91.21 (0.39)	91.67 (0.37)	91.52 (0.42)	91.36 (0.35)	90.93 (0.54)	91.50 (0.54)	92.12 (0.54)	91.23 (0.54)	0.400	0.771	0.186
Overall	75.50 (0.22)	75.87 (0.27)	76.08 (0.24)a	75.28 (0.23)b	75.62 (0.32)	75.37 (0.32)	76.54 (0.32)	75.2 (0.32)	0.259	0.019	0.098
Egg yolk redness											
Four	3.67 (0.12)	3.63 (0.15)	4.20 (0.04)a	3.10 (0.06)b	4.14 (0.07)a	3.19 (0.07)b	4.25 (0.07)a	3.65 (0.07)ab	0.581	0.000	0.046
Eight	4.01 (0.08)	4.31 (0.08)	4.23 (0.09)	4.08 (0.08)	4.14 (0.11)	3.89 (0.11)	4.33 (0.11)	4.28 (0.11)	0.014	0.194	0.401
Overall	3.84 (0.08)	3.97 (0.09)	4.22 (0.05)a	3.59 (0.06)b	4.14 (0.07)	3.54 (0.07)	4.29 (0.07)	3.64 (0.07)	0.094	0.000	0.733
Egg yolk yellowness											
Four	39.27 (0.32)	39.27 (0.36)	39.79 (0.25)a	38.76 (0.38)b	39.32 (0.44)ab	39.22 (0.44)ab	40.26 (0.44)a	38.29 (0.44)b	0.995	0.023	0.039
Eight	59.25 (0.41)	61.03 (0.97)	59.99 (0.96)	60.29 (0.53)	59.10 (1.08)	59.40 (1.08)	60.88 (1.08)	61.18 (1.08)	0.109	0.784	0.996
Overall	49.26 (0.31)	50.15 (0.52)	49.89 (0.53)	49.52 (0.32)	49.21 (0.61)	49.31 (0.61)	50.57 (0.61)	49.73 (0.61)	0.151	0.549	0.443

[1]Means followed by different letters in the same line differ significantly by Duncan's test ($p < 0.05$). [2]No suppl., no supplementation; and Suppl.; supplementation. [3]T1, low SD and no AA supplementation; T2, low SD and 150 mg of AA per kilogram of feed; T3, high SD and no AA supplementation; and T4, high SD and 150 mg of AA per kilogram of feed.

Table 5. Results of the mean (standard error) and p-value obtained for the blood components and stress indicators of Hy-line W-80 laying hens due to the main effects of L-ascorbic acid (AA) supplementation, stocking density (SD), and their interactions[1].

Blood[4]	SD		AA[2]		Treatment (interactions)[3]				p-value		
	Low	High	No suppl.	Suppl.	T1	T2	T3	T4	SD	AA	SD*AA
Glucose (mg dL^{-1})	162.2 (18.8)	190.8 (3.9)	184.2 (10.0)	168.0 (16.8)	180.0 (19.3)	188.5 (19.3)	144.4 (19.3)	193.2 (19.3)	0.146	0.428	0.303
Triglycerides (mg dL^{-1})	775.2 (132.7)b	1,203.2 (139.3)a	1,004.0 (123.7)	973.0 (162.8)	894.6 (194.5)	1,115.2 (194.5)	655.9 (194.5)	1,291.3 (194.53)	0.034	0.873	0.293
Cholesterol (mg dL^{-1})	103.5 (17.0)	103.3 (8.6)	100.0 (10.4)	106.0 (15.9)	107.8 (19.4)	93.6 (19.4)	99.3 (19.4)	113.1(19.4)	0.992	0.779	0.476
AST (mg dL^{-1})	158.1 (19.4)b	208.80 (7.2)a	191.0 (12.1)	175.0 (15.6)	175.4 (20.9)	207.1 (20.9)	140.8 (20.9)	210.5 (20.9)	0.020	0.460	0.369
ALT (mg dL^{-1})	3.30 (0.91)	2.50 (0.43)	2.60 (0.50)	3.20 (0.09)	3.4 (1.0)	1.8 (1.0)	3.2 (1.0)	3.2 (1.0)	0.437	0.559	0.437
Cortisol (µg L^{-1})	0.044 (0.005)b	0.054 (0.000)a	0.052 (0.004)	0.0046 (0.003)	0.05 (0.00)	0.05 (0.00)	0.04 (0.00)	0.05 (0.00)	0.035	0.281	0.281
Heterophils (%)	16.84 (34.38)	24.13 (34.38)	17.69 (2.32)	20.47 (2.18)	14.43 (2.93)	20.95 (3.59)	20.84 (3.21)	20.10 (2.93)	0.377	0.395	0.270
Lymphocytes (%)	23.10 (2.17)	25.40 (2.32)	23.79 (2.32)	24.71 (2.17)	22.95 (2.93)	24.63 (3.59)	23.24 (3.21)	26.18 (2.93)	0.477	0.775	0.844
Monocytes (%)	19.55 (1.90)	19.33 (2.02)	20.63 (2.02)	18.25 (1.90)	21.65 (2.56)	19.60 (3.13)	17.44 (2.80)	19.07 (2.56)	0.940	0.404	0.516
Eosinophils (%)	17.72 (2.26)	16.59 (2.41)	17.08 (2.41)	17.23 (2.260	16.97 (3.05)	17.20 (3.73)	18.48 (3.34)	15.98 (3.05)	0.736	0.965	0.684
Basophils (%)	21.97 (1.82)	18.10 (1.94)	20.76 (1.94)	19.31 (1.82)	23.95 (2.46)	17.58 (3.01)	19.98 (2.69)	18.63 (2.46)	0.166	0.592	0.359
H:L ratio	0.78 (0.11)	0.88 (0.12)	0.74 (0.12)	0.92 (0.110	0.65 (0.15)	0.83 (0.18)	0.91 (0.16)	0.93 (0.15)	0.549	0.270	0.616
Indicator					Stress						
Tonic immobility (s)	168.35 (34.38)	241.25 (34.38)	230.75 (34.38)	178.85 (34.38)	184.00 (48.61)	152.70 (48.61)	277.50 (48.61)	205.00 (48.61)	0.142	0.293	0.674
Respiratory rate (min)	65.58 (1.42)b	71.63 (1.51)a	67.96 (1.51)	69.25 (1.42)	63.17 (1.91)	72.75 (2.34)	68.00 (2.10)	70.50 (1.91)	0.010	0.542	0.106
Feather loss score	0.476 (0.07)b	0.899 (0.06)a	0.716 (0.06)	0.659 (0.06)	0.552 (0.10)	0.400 (0.10)	0.880 (0.07)	0.918 (0.08)	0.000	0.526	0.289

[1]Different letters in the same line differ significantly by Duncan's test ($p < 0.05$). [2]No suppl., no supplementation; and Suppl.; supplementation. [3]T1, low SD and no AA supplementation; T2, low SD and 150 mg of AA per kilogram of feed; T3, high SD and no AA supplementation; and T4, high SD and 150 mg of AA per kilogram of feed. [4]AST, aspartate aminotransferase; ALT, alanine aminotransferase; and H:L, heterophil:lymphocyte ratio.

glucose, triglycerides, and cholesterol in laying hens. However, Sahin et al. (2002) reported that the addition of L-ascorbic acid to hen diets increased serum protein but lowered serum glucose and cholesterol. In contrast, Osadcha et al. (2021) verified that a high SD did not significantly affect blood cholesterol levels. For Gholami et al. (2020), SD influenced the aspartate aminotransferase and alanine aminotransferase levels of hens.

In their study, Tactacan et al. (2009) observed that neither conventional cages with an area of 561.9 cm^2 nor enriched cages with an area of 642.6 cm^2 affected the levels of heterophils, lymphocytes, monocytes, eosinophils, or basophils in laying hens. Likewise, Kang et al. (2011) found that heterophils, lymphocytes, monocytes, and the heterophil:lymphocyte ratio remained unaffected by varying SD values, which ranged from 12 to 44 kg of body weight per square meter.

A higher SD was associated with elevated respiratory rates ($p < 0.05$) and feather loss scores across all evaluated periods ($p < 0.05$), indicating stress responses and possibly a decreased welfare under crowded conditions. Geng et al. (2020) also noted that a high SD tends to impair feather cover and lower the feather score. However, Widowski et al. (2017) and Tok et al. (2022) observed increased feather scores under a high SD.

Neither L-ascorbic acid supplementation nor SD had any effect on tonic immobility. However, in a previous study, Sanotra et al. (2001) found that broilers subjected to a high SD showed a significantly longer duration of tonic immobility. In another work under a similar context (Hrabcakova et al., 2012), pheasant hens housed in enriched cages remained in tonic immobility for a considerably shorter duration than those in conventional cages. Furthermore, Şekeroğlu et al. (2014) observed that a cage density of 600 cm^2 significantly increased tonic immobility in laying hens.

Conclusions

1. The addition of L-ascorbic acid to the diet of Hy-line W-80 laying hens does not eliminate the negative effects caused by the high stocking density on bird performance, egg quality, blood values, and feather score values.

2. Housing three laying hens per conventional cage enhances both yield and animal welfare parameters.

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No generative artificial intelligence (AI) was used in this study.

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The authors declare no conflicts of interest.

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