



■Author(s)

Tainika Bⁱ  <https://orcid.org/0000-0001-6785-4324>
Şekeroğlu Aⁱ  <https://orcid.org/0000-0002-1896-2449>
Akyol Aⁱ  <https://orcid.org/0000-0002-1009-2629>
Şentürk YEⁱ  <https://orcid.org/0000-0002-9218-5254>
Abacı SH^a  <https://orcid.org/0000-0002-1341-4056>
Duman Mⁱⁱ  <https://orcid.org/0000-0003-0342-8275>

ⁱ Department of Animal Production and Technologies, Faculty of Agricultural Sciences and Technologies, Niğde Ömer Halisdemir University, 51240, Niğde, Türkiye.

ⁱⁱ Department of Animal Science, Faculty of Agriculture, Ondokuz Mayıs University, 55139, Samsun, Türkiye.

ⁱⁱⁱ Department of Laboratory and Veterinary Health, Bor Vocational School, Niğde Ömer Halisdemir University, 51240, Niğde, Türkiye.

■Mail Address

Corresponding author e-mail address
Brian Tainika

Department of Animal Production and Technologies, Faculty of Agricultural Sciences and Technologies, Niğde Ömer Halisdemir University, 51240, Niğde, Türkiye.
Phone: +905522077723
Email: tainika2012b@yahoo.com

■Keywords

Aromatic plants; Egg quality traits; Free-range system; Laying hen.



Section editor: Tatiana Carlesso dos Santos

Submitted: 07/February/2024

Approved: 19/July/2024

Effects of Age, Housing Environment, and Strain on Physical Egg Quality Parameters of Laying Hens

ABSTRACT

This study examined the effects of age, housing environment, and strain (Lohmann Sandy (LS) and Lohmann LSL Classic (LW)) on egg quality traits. Deep litter (DL), free access to outdoor *Mentha piperita* (MP), *Petroselinum crispum* (PC), and *Medicago sativa* (MS) vegetated environments were examined. A total of 260 four-week-old birds were randomly distributed to DL and outdoor plant-associated groups, with four and three replicates, respectively, and 10 birds per replicate. Eggs were analyzed between 26 and 52 weeks of hen age, every 4 weeks. Overall, all egg quality parameters significantly differed as hen age increased ($p<0.01$). The housing environment significantly influenced egg weight, shell-breaking strength, shell thickness, egg surface area, and yolk color score. Eggs obtained from DL hens were heavier and had a higher egg surface area than those from MS, PC, and MP hens ($p<0.01$; $p<0.05$). However, eggs laid by MS, PC, and MP hens had higher shell-breaking strength and thickness ($p<0.01$; $p<0.05$). DL hens had a greater ratio of eggs with meat-blood inclusions in the yolk compared to MS, PC, and MP hens ($p<0.05$). Shape index, albumen height, albumen index, Haugh unit, and yolk index were similar among housing environments ($p>0.05$). Strain significantly affected shape index, shell thickness, albumen height, albumen index, Haugh unit, albumen pH, yolk index, and yolk color score. LS eggs had higher shape index, shell thickness, yolk index, and yolk color score ($p<0.01$; $p<0.05$). However, LW eggs had greater albumen height, albumen index, Haugh unit, and albumen pH ($p<0.01$; $p<0.05$). LW strain had a lower ratio of eggs with meat-blood inclusions compared to LS ($p<0.01$). Shell-breaking strength and egg surface area did not differ between hen strains ($p>0.05$). This study showed that allowing hens access to MS, PC, or MP plant species improved shell quality traits. Moreover, it appears that there is a greater genetic variability in albumen and yolk quality traits.

INTRODUCTION

Consumers in developed countries are increasingly interested in the welfare of laying hens, leading to an increase in demand for eggs from hens housed in environments without cages (Ochs *et al.*, 2018; Lusk, 2019). Free-range or organic systems are common types of cage-free systems, especially where birds have free access to different forage or plant materials in outdoor areas (Sossidou *et al.*, 2015; Hammershøj & Johansen, 2016). Furthermore, one of the significant developments in the egg layer industry is the debate on “end cage age” in the European Union parliament since 2018 (EFSA, 2023), whereby the ban on cage systems for laying hens will result in widespread cage-free production systems, including floor and free-range.

It is understood that egg quality is fundamental for consumer acceptability as well as increasing the total saleable eggs, which



determines the profitability of laying hen enterprises for the producers. Egg quality comprises various traits linked to the shell (external quality) and the albumen and yolk (internal quality) (Stadelman, 1977). Several egg quality traits vary with flock age (Samiullah *et al.*, 2014; Samiullah *et al.*, 2017; Sokołowicz *et al.*, 2018; Yılmaz Dikmen *et al.*, 2017; Yurtseven *et al.*, 2021; Şekeroğlu *et al.*, 2024), and can be modulated by the housing system (Sokołowicz *et al.*, 2018; Rakonjac *et al.*, 2014; Popova *et al.*, 2020; Dalle Zotte *et al.* 2021; Alig *et al.*, 2023).

Genetic structure has an effect on egg quality traits (Küçükyılmaz *et al.*, 2012; Batkowska & Brodacki, 2017; Rakonjac *et al.*, 2021). Moreover, there can be a genetic disparity in the plant intake, as well as the nutrient intake from different plants (Lorenz *et al.*, 2013). Thus, greater variability in some egg quality traits across laying hen strains with access to vegetated areas can be expected.

Studies suggest the possibility of enhancing egg quality traits with free access to outdoor pastures, especially with grass species (Hammershøj & Johansen, 2016). However, aromatic plants (herbs and species) have not been fully explored as a possibility to optimize free-range production systems and modulate egg quality in cage-free eggs. Nonetheless, the nutritional relevance of outdoor plant species is one of the main factors in determining the extent of modification of some egg quality traits (Horsted & Hermansen, 2007; Mugnai *et al.*, 2014; Dal Bosco *et al.*, 2016; Dalle Zotte *et al.*, 2021).

This study examined the quality traits of eggs obtained from two laying hen strains housed with or without free access to *Mentha piperita*, *Petroselinum crispum*, and *Medicago sativa* vegetated areas at different ages. It was hypothesized that granting hens free outdoor access to plant species would enhance egg quality compared to completely indoor rearing of hens. This could be linked to the intake of different plant species and their specific nutrients, other additional dietary resources, and the valuable environmental circumstances that come with outdoor housing. Furthermore, access to different outdoor aromatic plant species and legume pastures may happen to enhance the intake of different bioactive compounds in laying hens, which could result in variations of some egg quality traits.

Variation in egg quality traits between Lohmann LSL Classic and Lohmann Sandy would probably also occur based on our earlier studies that identified differences in the expression of foraging (Tainika *et al.* 2024a) and ranging (Tainika *et al.* 2024b) behaviors between the

two strains. Consequently, the physiological responses or mechanisms associated with egg quality traits would also differ as a result of differences in the behavioral activities between the strains.

MATERIALS AND METHODS

The animal experiments local ethics committee of Niğde Ömer Halisdemir approved this study (approval number: 2021/04). The study was carried out at the Ayhan Şahenk Agricultural Application and Research Centre of Niğde Ömer Halisdemir University, Niğde province, Türkiye (37°58' North 34°40'45 East, elevation; 1299 m).

Establishment of plant species

Each outdoor pen was ploughed, and stones were removed and leveled before the establishment of various plant species. The application of herbicides was not practiced during the land preparation. Plant species were then allocated randomly to the experimental replicate pens before their planting was initiated. The dimensions of the outdoor ranging area were 9.41 m × 1.94 m (total area = 18.25 m²).

Vegetative propagation using suckers or sprigs was used to cultivate *Mentha piperita*, which had been grown in pots in the greenhouse and then transported to the planting area after the complete establishment of the shoot system was achieved. The planting of suckers was done in rows and columns, approximately 10 cm apart to aid the establishment of a thick vegetative cover (200 suckers of *Mentha piperita* per m²).

Seeds were used in the establishment of *Petroselinum crispum* and *Medicago sativa*, using a seed rate of 183 g/ pen (10 g per m²) and 275 g/ pen (15 g per m²), respectively. The seeds were purchased from a certified seed seller in the province. Before sowing with the broadcasting method, 1 kg of artificial fertilizer was mixed with the seeds. Moreover, covering the pens for *Petroselinum crispum* was advised by a professional on forage plants to prevent the seeds from drying due to their sunlight sensitivity. Sowing was done in the evening for two straight days. Sprinkler irrigation was conducted twice a day (morning and evening), followed by flood irrigation once a day after sowing the seeds and suckers. This was practiced until full plant coverage of the pens was achieved.

Apart from rainy days and the entire winter season during the experimental period, watering was done every day in the evening after the pop holes were closed (3:30 pm) to maintain the vegetative stage of the plant species. With the exception of



weed removal and watering, the practice of other pasture management techniques such as rotational grazing, fertilizer application, and mowing were not implemented during the entire period when the birds were allowed access to the range.

Full plant coverage was achieved between August to September, and by estimation, almost 60% plant coverage in October and 30% coverage in November, with depletion expected due to the hens' activities. Almost no plant coverage was observed in December, and no plant coverage at all was present between January to February, apart from the standing stem parts (without leaves), due to the winter season. Plants began to grow again in March.

Birds, Housing, and Management

In the current study, two hundred sixty beak-trimmed Lohmann LSL Classic (LW) and Lohmann Sandy (LS) birds ($n = 130$ of each of the strains) were used. The popularity of these strains among local farmers was the reason they were chosen as experimental birds. The LW strain is a renowned strain characterized by an efficient production of white eggs, especially under the cage system. Although LS is currently not a popular strain among farmers, it is white-feathered, produces cream-colored eggs, and possesses a better feed conversion ratio and robustness (Lohmann, 2022).

Birds were purchased from a commercial breeder and brought to the experimental unit at 3 weeks of age. They were first housed in two litter floor pens based on their strain for 7 days, to acclimatize to the new environment. Birds were allocated randomly to one of the experimental replicate pens at 4 weeks of age. Each indoor pen measured 2.79 m^2 , with or without direct access to the outdoor pen (18.25 m^2) via a pop hole (50 cm high $\times$ 50 cm wide). The birds were housed in either the deep litter system without outdoor access (DL), and with access to outdoor ranging area cultivated either with *Mentha piperita* (MP), *Petroselinum crispum* (PC), or *Medicago sativa* (MS). For each strain, the number of replicates per housing environment was four for DL and three for outdoor plant-associated groups, each with a total of 10 birds. Overall, a total of 26 replicate pens and 260 birds were utilized for the study. All the housing environments were set on the same poultry house.

Until 11 weeks of age, all birds were reared indoors and wheat straw spread at a depth of 8 cm from the concrete floor was used as litter material in every indoor pen. During the experimental period, the litter was changed whenever it was reduced, and when caking was observed. Furthermore, each indoor pen

was equipped with a hanging feeder (diameter of 41 cm) and bell-type drinker (diameter of 30 cm) and later, a 3×3 (tier and cell) metallic nest box (98 cm $\times$ 37 cm $\times$ 138 cm) from 19 weeks of hens' age. However, the area occupied by the above equipment was not compensated for when determining the indoor stocking density, which was $3.58 \text{ birds} / \text{m}^2$.

The pens for the DL system were 16 in total, fixed in the centre of the poultry house and separated from each other by a wire mesh. However, only eight pens (four replicate pens per strain) close to the entrance and exit of the barn were used for this study. DL birds were kept completely indoors up to 52 weeks of age, the end of the study. The remaining eight DL pens located in the middle were left empty. The DL and plant-related housing environments were separated by a corridor almost three meters wide on both sides of the barn.

For the outdoor plant-based housing environments, the outdoor stocking density was $0.55 \text{ bird} / \text{m}^2$ (outdoor area of $1.825 \text{ m}^2 / \text{bird}$). Birds were granted range accessibility when they were 12 weeks old. Furthermore, all the pens were fenced and separated by wire mesh – although the outdoor pens had an open top. Due to the opened top, some hens ($n = 8$) could fly to the nearest pens; to prevent this, the flight feathers of one wing of only these 8 birds were trimmed. The barn used in this study lacked a veranda, and apart from the experimental plant species, other resources such as trees and shelters were not present in the vegetated outdoor pens. Before the birds were granted access to the outdoor plants, the mowing of the plant species was conducted to ensure that they were at a uniform height (20 cm).

The popholes were opened from 8:30 am and closed at 3:30 pm everyday. However, popholes remained closed for 8 days (d) after the birds had been vaccinated against fowlpox at 23 weeks of age (in October 2022), and the first three weeks of February 2023 (d 1 to 21) due to extreme cold and snowfall. The argument was that the welfare of hens could be significantly compromised.

Heating was provided by electric heaters in the first few weeks of the brooding period, with the internal temperature maintained at around 23°C to acclimatize the birds to the outside temperatures. Thereafter, the temperature fluctuated freely from 8 weeks of hen age until the end of the study. It should be emphasized that the study was conducted between June 2022 and May 2023, indicating a wider variation in the weather circumstances, especially temperatures. At the time the pop-holes were opened (12 weeks of hen



age), outdoor temperatures ranged from 23 - 35°C in August, and subsequently from 8 - 34°C between September to November, from 3 - 19°C between December to February, and from 9 - 25°C between March to May. Indoor temperatures ranged from 20.9 - 31°C degrees in August, from 5.6 - 30.3°C between September and November, from 1.1 - 15.1°C between December and February, and from 4 - 20.1°C between March and May.

Standard concentrate feed (purchased from a private company) was given to the birds in the current study, as shown in Table 1. Feed and water were offered to the birds *ad libitum*.

From 4 weeks of age, the birds were offered 13 hours of light, and a regular step-down program by Lohmann (2022) was followed until week 17 of age. Afterward, the photoperiod (light, L to dark, D) provided to birds was adjusted as follows: at 18 weeks: 10L:14D; 19 weeks: 10.30L:13.30D; 20 weeks: 11.15L:12.45D; 21 weeks: 12L:12D; 22 weeks: 12.45L:11.15D; 23 weeks: 13.30L:10.30D; and at the 24th week: 14L:10D. 16L:8D at 27 weeks of age was reached with the increment of the photoperiod by 30 min per week. After achieving 16L:8D at 27 weeks of age, the same photoperiod was used till the end of the study. The sources of light were warm, of white light LED bulbs of 14 watts / 2700 K.

A standard vaccination program was employed by the breeder firm until 3 weeks of age before the birds were brought to the experimental unit. Vaccination with Infectious Bronchitis + Newcastle (Ma5+Clone30) via drinking water and fowl pox vaccine (VAIOL-VAC) via the wing web at respectively 11 and 23 weeks of age were employed. Furthermore, the inclusion of a vitamin + amino acid premix in the drinking water after the vaccination process was carried out. Only 5 mortalities were recorded during the experimental period.

Egg quality analyzes

30% of the total eggs were collected (n = 78; 39 eggs per genetic strain; 3 eggs from each replicate) once at an interval of four weeks between 26 and 52 weeks of laying hen age. They were randomly selected, collected from each treatment replicate pen on the same day, and brought to the university laboratory for analysis. Quality analyzes were conducted after 24 hours of egg storage at room temperature. The analyzed egg quality traits were egg weight, shape index, shell strength, shell thickness, albumen height, albumen index, Haugh Unit, yolk index, yolk color, albumen pH, and blood-meat spots. The assessment criteria and formulas for egg quality traits are shown below.

First, the egg weight (g) was determined with a weighing scale with a precision of 0.001 g. This was followed by taking the egg length and width values with a digital caliper (± 0.001 mm), leading to the determination of the shape index (SI, %) as follows:

$$SI = (\text{Egg width} / \text{Egg length}) \times 100.$$

Subsequently, the shell-breaking strength (kg. force) was determined using an Orka food technology egg force device (Israel). The egg was then broken on a glass table to facilitate the evaluation of internal traits. During the process, albumen and yolk height were measured by a 1/100 mm micrometer, and the albumen length and width, and the yolk diameter were measured by a digital caliper (± 0.001 mm). This data was utilized to determine the traits indicated below:

$$\text{Yolk index, \%} = (\text{Yolk height/yolk diameter}) \times 100.$$

$$\text{Albumen index, \%} = (\text{Albumen height/ (albumen length + albumen width) / 2}) \times 100.$$

$$\text{Haugh unit,} = 100 \log (\text{albumen height} - 1.7 \text{ EW} 0.37 + 7.57) \text{ (Haugh, 1937)}.$$

Table 1 – Composition and ingredients of the commercial feeds used at various ages during the study.

Nutrient composition	Type of feed (age of hens)				
	Layer grower (3-8 weeks)	Layer developer (8-18 weeks)	Pre-peak lay (18-23 weeks)	Layer 1. phase (23-33 weeks)	Layer 2. phase (34 weeks until end of the study)
Crude protein, %	20.7	16	17.5	17	15.61
Crude cellulose, %	3.9	4.3	3.6	4.5	4.8
Crude ash, %	5.2	5.5	13.6	13.7	12.2
Crude fat, %	3.6	2.2	4.4	4.9	3.83
Calcium, %	0.2	1.2	3.9	3.9	3.83
Phosphorous, %	0.4	0.4	0.4	0.4	0.42
Sodium, %	3.9	0.2	0.1	0.1	0.16
Lysine, %	1	0.8	0.8	0.8	0.76
Methionine, %	0.5	0.4	0.4	0.4	0.37
Metabolic energy, Kcal/Kg	2700	2700	2700	2700	2700

Ingredients: Maize (produced from genetically modified maize), soya bean meal (produced from genetically modified soya), wheat, calcium carbonate, sunflower seed meal, Dried distillers grains (DDGS: produced from genetically modified maize), soya oil, dicalcium phosphate, sodium chloride, sodium bicarbonate; Vitamin A 12.000 IU; Vitamin D₃ 2.400 IU; Vitamin E 30 Mg / Kg; Mg 80 mg; Zn 60 mg; Cu 5 mg; Fe 60 mg; I 2 mg; Se 0.15 mg; Co 0.5 mg.



Moreover, albumen pH was determined by a manual pH meter (model 3310 545-007)/REV A/ 03-96, and the yolk color was scored using a DSM yolk color fan, with the density of color ranging from pale yellow to dark orange, represented on a scale of 1 to 15 (DSM Nutritional Products Ltd, 2013).

Additionally, shell thickness (mm) was calculated as the average of three shell thickness values taken from the center, broad, and sharp points of the egg. Shell thickness was measured by a 1/100 mm micrometer, without the shell membrane.

Egg weight values were also used to calculate the egg surface area as follows:

Egg surface area, cm²: = 3.9782 × egg weight in grams^{0.70} (Carter, 1975).

Blood and meat inclusions in the albumen and yolk were determined by a visual inspection and the data was later segregated as percentage of eggs with meat or blood inclusions in the egg compartments out of the total analyzed eggs per replicate.

Statistical analysis

During the data analysis, the effect of the factors on egg quality traits was examined. The normality assumptions of the data were examined using the Kolmogorov-Smirnov test, and tests for homogeneity of variance were conducted using the Levene test,

confirming that the assumptions were met. Accordingly, an analysis of variance was applied to the data. Intra-group multiple comparisons were conducted using Duncan's multiple comparison test at $p < 0.05$. The statistical software package SPSS 21 was used for data analysis. The following statistical model was used for the analysis of the data:

First model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \theta_k + \alpha\beta_{ij} + \alpha\theta_{ik} + \beta\theta_{jk} + \alpha\beta\theta_{ijk} + \varepsilon_{ijkl}, \varepsilon_{ijkl} \sim (0, \sigma^2)$$

In the model, Y_{ijkl} : observation value, μ : population mean, α_i : the effect of i. age, β_j : the effect of the j. housing environment, θ_k : the effect of i. strain, $\alpha\beta_{ij} + \alpha\theta_{ik} + \beta\theta_{jk} + \alpha\beta\theta_{ijk}$: interaction effects and ε_{ijkl} : random error $\varepsilon_{ijkl} \sim N(0, \sigma^2)$.

RESULTS

The results for egg weight, shape index, egg surface area, shell breaking strength, and shell thickness are shown in Table 2. Eggs became heavier with the aging of hens ($p < 0.01$), and DL hens laid heavier eggs than MS, PC, and MP hens ($p < 0.01$). However, egg weight was not different between hen strains ($p > 0.05$). There was significant hen age × housing environment interaction for egg weight (Table 3; $p < 0.01$).

Table 2 – Effect of age (between 26 and 52 weeks of age), housing environment, and hen strain on external egg quality traits.

Factor		Egg weight (g)	Shape index (%)	Shell breaking strength, (kg. f)	Shell thickness, (mm)	Egg surface area (cm ²)
Age of hens (A, week)	26	56.64 ^e	78.06 ^{ab}	5.41 ^{ab}	0.407 ^b	67.12 ^e
	30	59.97 ^d	78.30 ^a	5.19 ^{bc}	0.407 ^b	69.85 ^d
	34	62.52 ^c	77.24 ^{bc}	5.43 ^a	0.414 ^a	71.92 ^c
	38	62.74 ^c	77.63 ^{ab}	5.38 ^{ab}	0.408 ^{ab}	72.09 ^c
	42	63.16 ^{bc}	75.43 ^d	5.22 ^{a-c}	0.365 ^c	72.43 ^{bc}
	46	63.70 ^{ab}	76.73 ^c	5.03 ^{cd}	0.267 ^d	72.87 ^{ab}
	50	64.27 ^a	76.44 ^c	4.94 ^d	0.251 ^e	73.32 ^a
	p value	<0.001	<0.001	<0.001	<0.001	<0.001
HE	DL	62.52 ^a	77.45	5.06 ^b	0.356 ^b	71.91 ^a
	PC	61.44 ^b	76.85	5.25 ^a	0.362 ^a	71.04 ^b
	MP	61.62 ^b	77.09	5.32 ^a	0.359 ^{ab}	71.18 ^b
	MS	61.61 ^b	76.98	5.35 ^a	0.363 ^a	71.18 ^b
	p value	<0.001	0.208	<0.001	<0.008	<0.001
Hen strain (HS)	LS	62.00	77.93	5.25	0.362	71.49
	LW	61.71	76.44	5.21	0.357	71.26
	p value	0.160	<0.001	0.692	<0.012	0.156
	SEM	0.143	0.120	0.029	0.001	0.116
	A × HE	<0.003	<0.035	0.220	<0.001	<0.003
	A × HS	0.654	0.238	0.292	<0.001	0.653
	HE × HS	0.618	0.980	0.119	0.345	0.642
	A × HE × HS	0.693	0.798	0.441	<0.001	0.691

Abbreviations: HE: housing environment, DL: deep litter, PC: *Petroselinum crispum*, MP: *Mentha piperita*, MS: *Medicago sativa*, LS: Lohmann Sandy, LW: Lohmann LSL Classic, SEM: standard error of mean, ×: interactions between different factors. Means within the same column with different letter superscripts significantly differ ($p < 0.05$).



The shape index significantly decreased as hen age increased ($p<0.01$), with the lowest decline in eggs at 42-weeks of age. The shape index was significantly higher in eggs obtained from LS as compared to the LW strain ($p<0.01$), but was not different among the housing environments ($p>0.05$). There was a significant hen age $\times$ housing environment interaction for shape index (Table 3; $p<0.05$).

There was a clear increasing trend for egg surface area as hen age increased ($p<0.01$), being highest and lowest at 50 and 26 weeks of age, respectively. Egg surface area differed among the housing environments ($p<0.01$), being higher in eggs from DL hens than in those from the MS, PC, and MP environments. No strain effect was identified for egg surface area ($p>0.05$). The age $\times$ housing environment interaction effect was observed on the egg surface area (Table 3; $p<0.01$).

Regarding shell quality traits, shell-breaking strength and shell thickness significantly decreased as

hen age increased ($p<0.01$). However, eggs with the highest shell-breaking strength and shell thickness were obtained when hens were 34 weeks old. MS, PC, and MP hens laid eggs with stronger and thicker shells than DL hens ($p<0.01$). Additionally, shell thickness varied among outdoor plant housing environments, being lower in eggs obtained from MP than PC and MS hens. While there was no variation between hen strains for shell-breaking strength ($p>0.05$), there was a significant variation between hen strains for shell thickness; which was higher in eggs from the LS than the LW strain ($p<0.05$). Lastly, there was significant age $\times$ housing environment, age $\times$ strain, and age $\times$ housing environment $\times$ strain interaction effects on shell thickness ($p<0.01$). However, the effect of age $\times$ housing environment interaction on shell thickness is shown in Table 3.

The results for internal egg quality parameters, that is albumen and yolk quality traits, are indicated in Table

Table 3 – Effect of age of hens $\times$ housing environment interaction on egg weight, shape index, egg surface area, and shell thickness.

Age of hens (week)	Housing environment	Egg weight (g)	Shape index (%)	Egg surface area (cm ²)	Shell thickness (mm)
26	DL	56.80 ^k	77.41 ^{ab}	67.25 ^k	0.402 ^{bc}
	PC	57.06 ^k	77.29 ^{ab}	67.46 ^k	0.410 ^{ab}
	MP	55.39 ^l	77.97 ^a	66.07 ^l	0.407 ^{ab}
	MS	57.27 ^k	77.40 ^{ab}	67.64 ^k	0.410 ^{ab}
30	DL	59.96 ^{ij}	76.75 ^{a-c}	69.84 ^{ij}	0.408 ^{ab}
	PC	59.52 ^j	76.60 ^{a-c}	69.48 ⁱ	0.402 ^{bc}
	MP	60.07 ^{h-j}	76.68 ^{a-c}	69.94 ^{h-j}	0.411 ^{ab}
	MS	60.33 ^{h-j}	75.56 ^{bc}	70.15 ^{h-j}	0.407 ^{ab}
34	DL	63.63 ^{a-e}	77.82 ^a	72.81 ^{a-e}	0.410 ^{ab}
	PC	61.54 ^{gh}	78.40 ^a	71.13 ^{gh}	0.412 ^{ab}
	MP	62.23 ^{a-g}	78.46 ^a	71.69 ^{e-g}	0.415 ^{ab}
	MS	62.33 ^{a-g}	77.65 ^{ab}	71.77 ^{d-g}	0.422 ^a
38	DL	64.28 ^{a-c}	77.10 ^{a-c}	73.34 ^{ab}	0.407 ^{ab}
	PC	61.32 ^{g-i}	76.34 ^{a-c}	70.95 ^{g-i}	0.411 ^{ab}
	MP	62.87 ^{b-g}	75.08 ^c	72.20 ^{b-g}	0.415 ^{ab}
	MS	61.91 ^{fg}	76.78 ^{a-c}	71.42 ^{fg}	0.401 ^{bc}
42	DL	64.62 ^a	77.85 ^a	73.61 ^a	0.357 ^e
	PC	62.83 ^{c-g}	77.52 ^{ab}	72.16 ^{b-g}	0.374 ^d
	MP	62.10 ^{e-g}	77.95 ^a	71.58 ^{e-g}	0.346 ^e
	MS	62.60 ^{d-g}	76.85 ^{a-c}	71.98 ^{c-g}	0.388 ^c
46	DL	63.96 ^{a-d}	76.94 ^{a-c}	73.08 ^{a-d}	0.264 ^{f-h}
	PC	63.37 ^{a-f}	76.41 ^{a-c}	72.60 ^{a-f}	0.274 ^f
	MP	64.21 ^{a-d}	77.68 ^{ab}	73.27 ^{a-c}	0.270 ^{fg}
	MS	63.19 ^{a-f}	76.30 ^{a-c}	72.47 ^{a-f}	0.262 ^{f-h}
50	DL	64.43 ^{a-c}	76.98 ^{a-c}	73.45 ^{ab}	0.244 ⁱ
	PC	64.46 ^{a-c}	76.83 ^{a-c}	73.47 ^{ab}	0.251 ^{hi}
	MP	64.49 ^{ab}	76.63 ^{a-c}	73.50 ^{ab}	0.252 ^{hi}
	MS	63.64 ^{a-e}	77.75 ^{ab}	72.82 ^{a-e}	0.258 ^{g-i}
SEM		0.143	0.120	0.116	0.298
p value		<0.003	<0.035	<0.003	<0.001

Abbreviations: DL: deep litter, PC: *Petroselinum crispum*, MP: *Mentha piperita*, MS: *Medicago sativa*, SEM: standard error of mean. Means within the same column with different letter superscripts significantly differ ($p<0.05$).



4. For albumen quality traits, there was a significant decrease in albumen height, albumen index, and Haugh unit; however, there was a significant increase in albumen pH as the hen age increased ($p < 0.01$). The lowest value for albumen height and Haugh unit was at 42 and 46 weeks of age, and for albumen index, it was at 42 weeks of age. The highest value for albumen

pH was at 46 weeks of age. No albumen quality traits differed among the housing environments ($p > 0.05$). The strain influenced all the albumen quality traits: they were higher in eggs obtained from LW than the LS strain ($p < 0.01$). Also, there was a significant age $\times$ strain interaction effect on albumen pH (Table 5; $p < 0.01$).

Table 4 – Effect of age (between 26 and 52 weeks of age), hen strain, and housing environment on internal egg quality traits.

Factor		Albumen height (mm)	Albumen index (%)	Haugh Unit	Yolk index (%)	Yolk color score (DSM)	Albumen pH
Age of hens (A, week)	26	9.53 ^a	13.09 ^a	97.71 ^a	48.08 ^a	13.78 ^a	8.85 ^f
	30	9.19 ^b	12.14 ^b	95.30 ^b	46.44 ^b	13.79 ^a	8.95 ^e
	34	9.13 ^b	11.92 ^b	94.38 ^b	47.84 ^a	12.97 ^{bc}	9.08 ^c
	38	9.20 ^b	11.96 ^b	94.71 ^b	48.09 ^a	13.14 ^b	9.04 ^d
	42	7.44 ^d	9.52 ^d	84.83 ^d	42.49 ^d	11.92 ^e	9.19 ^b
	46	7.70 ^d	10.08 ^c	86.13 ^d	43.56 ^c	12.92 ^c	9.26 ^a
	50	8.03 ^c	10.15 ^c	87.98 ^c	42.61 ^{cd}	12.63 ^d	9.21 ^b
	<i>p</i> value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
HE	DL	8.64	11.24	91.55	45.51	13.07 ^a	9.10
	PC	8.61	11.20	91.77	45.40	13.10 ^a	9.10
	MP	8.46	11.14	90.80	45.33	12.91 ^b	9.08
	MS	8.69	11.45	92.13	46.08	12.98 ^b	9.05
	<i>p</i> value	0.256	0.438	0.257	0.190	<0.034	0.054
Hen strain (HS)	LS	8.25	10.68	89.67	46.43	13.08	9.06
	LW	8.95	11.84	93.46	44.71	12.96	9.10
	<i>p</i> value	<0.001	<0.001	<0.001	<0.001	<0.014	<0.002
SEM		0.056	0.090	0.321	0.172	0.037	0.009
A $\times$ HE		0.471	0.678	0.525	<0.033	0.083	0.429
A $\times$ HS		0.958	0.882	0.814	<0.030	0.274	<0.001
HE $\times$ HS		0.750	0.654	0.707	0.126	<0.008	0.670
A $\times$ HE $\times$ HS		0.960	0.904	0.943	<0.013	<0.007	0.611

Abbreviations: HE: housing environment, DL: deep litter, PC: *Petroselinum crispum*, MP: *Mentha piperita*, MS: *Medicago sativa*, LS: Lohmann Sandy, LW: Lohmann LSL Classic, SEM: standard error of mean, $\times$: interaction between different factors. Means within the same column with different letter superscripts significantly differ ($p < 0.05$).

Table 5 – Effect of the age $\times$ strain interaction on albumen pH and yolk index (%).

Age of hen (week)	Strain	Albumen pH	Yolk index (%)
26	LS	8.86 ^{ij}	47.83 ^{a-c}
	LW	8.84 ⁱ	48.35 ^{a-c}
30	LS	8.98 ^{gh}	47.41 ^{bc}
	LW	8.92 ^{hi}	45.47 ^{de}
34	LS	9.09 ^{c-e}	48.53 ^{ab}
	LW	9.08 ^{d-e}	47.11 ^{bc}
38	LS	9.01 ^{fg}	49.29 ^a
	LW	9.07 ^{ef}	46.85 ^{cd}
42	LS	9.14 ^{cd}	44.06 ^{e-g}
	LW	9.24 ^b	40.93 ⁱ
46	LS	9.22 ^b	44.49 ^{ef}
	LW	9.31 ^a	42.65 ^{gh}
50	LS	9.15 ^c	43.35 ^{f-h}
	LW	9.27 ^{ab}	41.87 ^{hi}
SEM		0.009	0.172
<i>p</i> value		<0.001	<0.030

Abbreviations: LS: Lohmann Sandy, LW: Lohmann LSL Classic, SEM: standard error of mean. Means within the same column with different letter superscripts significantly differ ($p < 0.05$).

For yolk quality traits, yolk index and color significantly decreased as the hen age advanced ($p < 0.01$). However, the lowest value for both traits was at 42 weeks of age. While the yolk index was not significantly different across the housing environment ($p > 0.05$), that was not the case for yolk color ($p < 0.05$), which was darker in eggs from DL and PC than MP and MS, both groups with similar values. Yolk index and color significantly differed between strains ($p < 0.01$), being higher in eggs from the LS than from the LW strain. There was a significant effect of the age $\times$ housing environment $\times$ strain interaction on both traits ($p < 0.01$). In addition, a significant effect of the age $\times$ strain interaction ($p < 0.01$; Table 5) and the age $\times$ housing environment interaction ($p < 0.01$; Table 6) was observed on the yolk index.

Table 7 shows the data for meat and blood inclusions in the egg compartments. While the ratio of eggs with meat-blood spots in the albumen was significantly impacted by the age of hens ($p < 0.05$), being highest



Table 6 – Effect of the hen age × housing environment interaction on yolk index (%).

Age of hen, week	Housing environment	Yolk index (%)
26	DL	49.18 ^a
	PC	47.99 ^{a-c}
	MP	46.78 ^{a-d}
	MS	48.07 ^{a-c}
30	DL	46.71 ^{a-e}
	PC	46.31 ^{c-f}
	MP	46.51 ^{b-f}
	MS	46.14 ^{c-f}
34	DL	47.07 ^{a-c}
	PC	48.15 ^{a-c}
	MP	47.53 ^{a-c}
	MS	48.85 ^{ab}
38	DL	47.26 ^{a-c}
	PC	47.79 ^{a-c}
	MP	48.52 ^{a-c}
	MS	49.10 ^a
42	DL	41.18 ^j
	PC	42.68 ^{g-i}
	MP	42.19 ^{g-i}
	MS	44.37 ^{e-g}
46	DL	44.16 ^{f-h}
	PC	42.03 ^{g-i}
	MP	43.40 ^{g-i}
	MS	44.43 ^{d-g}
50	DL	43.22 ^{g-i}
	PC	42.98 ^{g-i}
	MP	42.26 ^{g-i}
	MS	41.78 ^{hi}
SEM		0.172
p value		0.033

Abbreviations: DL: deep litter, PC: *Petroselinum crispum*, MP: *Mentha piperita*, MS: *Medicago sativa*, SEM: standard error of mean. Means within the same column with different letter superscripts significantly differ ($p < 0.05$).

Table 7 – Effect of age, strain, and housing environment on the percentage of eggs with meat and blood inclusions in the albumen and yolk.

Factor		% of eggs with meat-blood inclusions in the albumen	% of eggs with meat-blood inclusions in the yolk
Age of hen (A, week)	26	12.82 ^{ab}	2.56
	30	14.10 ^{ab}	5.13
	34	6.41 ^b	5.13
	38	10.26 ^{ab}	3.85
	42	16.67 ^{ab}	6.41
	46	20.51 ^a	2.56
	50	6.41 ^b	7.69
	P value	<0.049	0.771
Housing environment (HE)	DL	13.10	7.74 ^a
	PC	9.52	2.38 ^b
	MP	11.90	3.97 ^{ab}
	MS	15.08	3.97 ^{ab}
	P value	0.566	<0.047
Hen strain (HS)	LS	22.71	8.42
	LW	2.20	1.10
	P value	<0.001	<0.001
SEM		1.565	0.905
A × HE		0.634	0.064
A × HS		0.104	0.969
HE × HS		0.129	0.056
A × HE × HS		0.868	0.113

Abbreviations: DL: deep litter, PC: *Petroselinum crispum*, MP: *Mentha piperita*, MS: *Medicago sativa*, LS: Lohmann Sandy, LW: Lohmann LSL Classic, SEM: standard error of mean, ×: interactions between different factors.

previously reported (Samiullah *et al.*, 2016; Samiullah *et al.*, 2017; Sokołowicz *et al.*, 2018; Şekeroğlu *et al.*, 2024).

The present study found heavier eggs for hens in deep litter than in outdoor plant housing environments, which is consistent with the findings of Islam *et al.* (2021). Generally, the increase in hen body weight has been linked to increase in nutrient intake, leading to the increase in egg weight (Leeson & Summers, 1987). It is speculated that due to the reduced space for energy-demanding movements and behavioral activities for hens housed indoors compared to the free-range hens, the indoor hens would have the advantage of depositing more nutrients in eggs as compared to the free-range hens, which may conserve more energy for other activities, such as foraging. However, contradicting results on egg weight have been previously reported. For instance, there are reports of heavier eggs being laid by free-range compared to deep litter hens (Sokołowicz *et al.*, 2018; Popova *et al.*, 2020; Rakonjac *et al.*, 2021); housing systems having no effect on egg weight (Mugnai *et al.* 2009); and similar egg weights in hens granted access to either non-vegetated, chicory and/or white

at 46 weeks of age and lowest at both 34 and 50 weeks of age; the opposite was true for the ratio of eggs with meat-blood inclusions in the yolk ($p > 0.05$). The housing environment effect was identified on the ratio of eggs with meat-blood spots in the yolk ($p < 0.05$), being highest in DL hens and lowest in PC hens. However, there was no housing environment effect on the ratio of eggs with meat-blood inclusions in the albumen ($p > 0.05$). There was a strain influence on the ratio of eggs with meat-blood inclusions in the albumen and ratio of eggs with meat-blood inclusions in the yolk ($p < 0.01$), which were both higher in the LS than the LW strain.

DISCUSSION

Similar to the current findings, age-related changes in egg weight, and in particular, the increase in egg weight with increasing age of hens have been



clover vegetated areas (Kop-Bozbay *et al.*, 2021). The difference between the findings of the current study and other studies may be related to the strain, type of housing environment, type of outdoor pasture plant, and age of hens.

In the present study, the lack of strain differences in egg weight contrasts with studies that reported genetic influences on egg weight (Steenfeld & Hammershoj, 2015; Lordelo *et al.*, 2020; Rakonjac *et al.*, 2021). A statistically significant interaction between the housing environment $\times$ age of hens could indicate that a pattern of change in egg weight with the aging of hens differed among housing environments (Singh *et al.*, 2009; Samiullah *et al.*, 2014).

Hen age did not significantly affect shape index in previous studies (Şekeroğlu *et al.*, 2014; Sokołowicz *et al.*, 2018), contrary to the present study. The significant difference in shape index in terms of hen age could be a direct effect of the larger eggs being laid by older hens compared to the smaller eggs laid by younger birds. The current results showing no housing environment effect on shape index are in agreement with several reports (Popova *et al.*, 2020; Rakonjac *et al.*, 2021). On the other hand, they disagree with the findings of Sokolowicz *et al.* (2018), who determined a higher shape index in eggs from the litter floor as compared to those of free-range and organic systems.

In this study, the shape index was higher in eggs from the LS than from the LW strain, indicating a genetic influence on shape index, as identified by several other studies (Sokołowicz *et al.*, 2018; Lordelo *et al.*, 2020; Rakonjac *et al.*, 2021; Sözcü *et al.*, 2021). A statistically significant interaction effect between age and housing environment on egg shape index is probably associated with the pattern of change in shape index with increase in age of hens across housing environments (Singh *et al.*, 2009; Samiullah *et al.*, 2014). The reason for this phenomenon is difficult to understand. However, Travel *et al.* (2011) linked age-associated variations in shape index to the weakening of the muscular tone of the shell gland and a change in the ratio of thick to thin albumen. Additionally, the environmental circumstances (temperature, airflow, and humidity) corresponding to seasonal changes influence egg weight loss due to evaporation from the time an egg is laid (Feddern *et al.*, 2017). This would result in a pattern of changes in the ratio between egg length and width, the traits used to calculate shape index across the age of hens during the study, which aligned with seasonal changes.

In the present study, egg surface area increased with the increase in the age of hens. A similar effect was reported by Şekeroğlu *et al.* (2014). Egg surface area was also higher in eggs from deep litter than in those from the outdoor plant housing environments. This is not in agreement with Ahmad *et al.* (2019) and Şekeroğlu & Sarıca (2005), who identified similar egg surface area in eggs obtained from hens housed in indoor and outdoor systems. Again, since access to outdoor plant species depressed egg weight, these results of egg surface area were expected. In the current study, egg surface area was similar between strains, which is contrary to Ahmad *et al.* (2019) and Şekeroğlu & Sarıca (2005), who reported a genetic influence on the egg surface area. The variation in the results could be attributed to the differences in the strains that were used in the studies.

Shell breaking strength and shell thickness are well-established shell quality traits, and the age-related effect on these traits was demonstrated by some studies (Samiullah *et al.*, 2017; Sokołowicz *et al.*, 2018), which is consistent with the findings of the present study. In this study, the shell quality traits worsened from 38 and 52 weeks of age. This could be associated with the reduction in the ability to deposit the required amount of calcium and phosphorus for eggshell formation as a result of weakened gastrointestinal tracts due to aging, thus resulting in hens losing their ability to digest and absorb nutrients. Interestingly, Şekeroğlu *et al.* (2014) found improved shell quality traits with the aging of Atak-S hens in enriched cages.

In the present study, eggs with superior shell quality traits were laid by hens in outdoor plant housing environments, indicating that these plants might contain specific bioactive compounds that enhance intestinal calcium absorption mechanisms, leading to stronger shells. Furthermore, it could be argued that access to these plant species might be associated with additional calcium intakes. Additionally, free-range hens scavenge and feed on various insects, which are a rich additional source of calcium.

However, this would contradict the findings of Kop-Bozbay *et al.* (2021), who reported similarity in shell-breaking strength and variation in shell thickness of eggs obtained from hens reared with access to non-vegetated, chicory and/or white clover-vegetated areas. The type of outdoor plants or lack of outdoor plants may have accounted for the differences in the results of the current study as compared to others.

The study by Sokolowicz *et al.* (2018) reported a genetic influence on shell thickness, but not on shell-



breaking strength, which is in line with the present study. In contrast, Ahmad *et al.* (2019) found no genetic influence on the shell thickness of eggs obtained from crosses of Rhode Island Red × Naked neck and the Black Australorp × Naked neck hens reared in semi-intensive and intensive systems. The statistically significant interaction effects on eggshell thickness could reflect the pattern of variations in shell thickness between strains across the housing environments and at different ages.

It is well-known that albumen height, albumen index, Haugh unit, and albumen pH constitute the albumen quality traits. In the present study, while albumen height, albumen index, and Haugh unit showed a relatively declining trend as hens aged, albumen pH showed the opposite pattern. A large percentage of the albumen is water from the hen's body; however, as hens age, they lose the ability to deposit a large percentage of nutrients in eggs due to the weakening of various body systems and organs, which accounts for the lower albumen traits with increasing hen age. Also, the results of the present study are in line with studies that demonstrated changes in albumen quality traits with the aging of hens. For example, reports of decreased albumen index and Haugh unit and increased albumen pH (Şekeroğlu *et al.*, 2014), decreased albumen height (Singh *et al.* 2009), and decreased Haugh unit (Sokolowicz *et al.*, 2018).

In the present study, all the albumen quality traits were similar across the housing environments. It can be argued that the conditions of the housing environments did not vary a lot, and thus hens' sensitivity to the environmental conditions did not impact egg production. Thus, the degree of deterioration in albumen quality due to the specific environmental circumstances was also relative. Moreover, this would be in accordance with some studies that identified similar Haugh unit and albumen index in eggs from hens that accessed either non-vegetated, chicory and/or white clover-vegetated areas (Kop-Bozbay *et al.*, 2021), and similar albumen height and Haugh unit in eggs obtained from hens housed in floor and organic systems (Rakonjac *et al.*, 2021). On the contrary, several studies have reported housing system effects on albumen quality traits; with reports of lower albumen index and Haugh unit in eggs obtained from free-range as compared to deep litter systems (Popova *et al.*, 2020), higher albumen index and Haugh unit in eggs obtained from deep litter than free-range hens (Şekeroğlu & Sarica, 2005), and higher albumen pH in eggs obtained from deep litter than free-range systems (Şekeroğlu *et al.*, 2010).

In this study, there was a genetic influence on all the albumen quality traits, whereby all the albumen quality traits were lower in LS as compared to LW eggs. Poggenpoel (1986) identified a negative correlation between the number of eggs produced and albumen quality. Indeed, in our earlier report (Tainika *et al.*, 2024c), daily egg production was significantly higher in hens of the LS than in the LW strain. Also, the selection for albumen quality might be more advanced in some strains than others, which is linked to the argument of Wan *et al.* (2019) that the selection for the thick-to-thin albumen ratio could be advantageous to enhance egg albumen quality. Furthermore, the present experiment confirms the results of several other studies. For instance, genetic influence was identified on albumen height (Singh *et al.*, 2009), albumen index and Haugh unit (Sözcü *et al.*, 2021), and Haugh unit and albumen pH (Lordelo *et al.*, 2020). Additionally, different strains may vary in terms of body weight and blood biochemistry, which could give rise to a variation in the ability to deposit, and the total amount of nutrients that could be deposited in eggs, thus influencing albumen traits such as pH.

Yolk index and color are also important yolk quality traits. In the current study, the unstable decreasing trend in the yolk index with the aging of birds contrasts with Sekeroglu *et al.* (2014), who identified a clear decreasing trend in the yolk index with the aging of birds. Moreover, the unclear trend of age effect on yolk color score possibly reflects the pattern of changes in outdoor plant coverage and range use and the consequent variation in plant intake across the seasons, as reported by Sokolowicz *et al.* (2018).

A previous study by Kop-Bozbay *et al.* (2021) reported no significant effect of housing environment, access to non-vegetated, chicory and/or white clover-vegetated areas on yolk index, which is in agreement with the current findings. In the present study, when the plant housing environments are compared, it could be argued that the concentration of carotenoid pigments for yolk color was higher in PC than in other plant-associated groups. However, both DL and PC groups had a similar value of yolk color score, which contrasts with previous studies that found darker yolk color in eggs from free-range systems than from deep litter hens (Şekeroğlu & Sarica, 2005; Sokolowicz *et al.*, 2018). On the other hand, the yolk color score was similar in eggs from hens permitted access to non-vegetated, chicory and/or white clover-vegetated areas (Kop-Bozbay *et al.*, 2021).



In the present study, the yolk index and color varied between strains, showing the genetic influence on both traits. This is consistent with previous studies (Küçükyılmaz *et al.*, 2012; Sokolowicz *et al.*, 2018; Sözcü *et al.*, 2021) that identified genetic effect on yolk color. In contrast, studies by Şekeroğlu & Sarıca (2005) and Ahmad *et al.* (2019) reported no strain difference in relation to the yolk index. The genetic difference in terms of yolk color could be associated with the different gastrointestinal tract efficiencies among strains, which influence the capacity for deposition of yolk color pigment in eggs.

In the present study, the unclear trend of age effect on yolk color score was possibly due to the seasonal changes that came with the pattern of changes in the outdoor vegetation coverage across the months, and these circumstances aligned with the aging of birds. For instance, plant coverage was high between 26 to 30 weeks of hen age, corresponding to a higher yolk color score, and almost no plant coverage was available during winter months (42 weeks), corresponding to the lowest yolk color score. This would be in line with Sokolowicz *et al.* (2018), who identified more intense yolk color in eggs from free-range hens at the beginning and end of their study, corresponding to autumn and spring, when green forage was plentiful; and less intense yolk color in eggs during the winter months, possibly due to less time spent outside and the unavailability of green forage.

However, several reports suggest that what hens consume influences yolk color score rather than the age of hens (Hammershøj & Steenfeldt 2012; Steenfeldt & Hammershøj, 2015), but dietary effects may be time-dependent in relation to the deposition of yellow pigments into the yolk (Hammershøj & Steenfeldt 2005). Again, this might be the possible explanation for the current results of the housing system and age effect on yolk color score.

The present study demonstrated that meat-blood spots in eggs significantly varied at different ages. Nalbandov & Card (1944) reported that blood spots in eggs originate from hemorrhages before ovulation, and meat spots occur after the transformation of blood spots after the eggs are laid, catalyzed by high environmental temperature. However, the results of the present study seem to indicate that the aging of birds might result in a variation in the above mechanisms. In addition, the results of the present study partially agree with Sokolowicz *et al.* (2018), who identified a significant effect of age on blood spots, but no effect on meat spots in eggs. Additionally, some earlier studies

had also indicated the age effect on meat and blood inclusions in eggs (Nalbandov & Card, 1944; Jeffrey, 1945; Jensen *et al.*, 1952). In contrast, Hammershøj *et al.* (2021) identified no hen age effect on blood and meat spots in eggs.

Furthermore, in the present study, a lower ratio of eggs with meat-blood spots in outdoor-based environments can indicate that the present plant species might contain specific nutritional factors associated with averting follicular hemorrhages (Nalbandov & Card, 1947), one of the causes of meat-blood spots in eggs. However, there are also contradictory reports on meat-blood spots in eggs. For instance, Sokolowicz *et al.* (2018) did not confirm meat-blood inclusions in eggs obtained from different alternative production systems, and the ratio of meat-blood spotted eggs did not differ between deep litter and free-range systems (Şekeroğlu & Sarıca, 2005). However, the ratio of meat-blood spotted eggs was higher in free-range than in deep litter hens (Şekeroğlu *et al.*, 2010).

In the present study, the ratio of eggs with meat-blood spots was higher in LS hens than in LW hens, which confirms earlier reports that the tendency to lay eggs with blood spots has a hereditary basis (Nalbandov & Card, 1944; Jeffrey, 1945; Campo & Gil, 1998; Hammershøj *et al.*, 2021). Moreover, Şekeroğlu & Sarıca (2005) demonstrated the genetic influence on meat-blood spots, higher in eggs from brown than white layers.

CONCLUSIONS

This study suggested that access to MS, PC, and MP seemed appropriate for increasing the shell-breaking strength and thickness, but the DL system is ideal for heavier eggs. However, the DL system might be related to increased meat and blood inclusions in the eggs. Additionally, the LS and LW eggs might be associated with better yolk and albumen quality traits, respectively. Nonetheless, the LS strain is linked to greater meat and blood inclusions in egg compartments, suggesting greater genetic disparity in meat and blood inclusions in eggs.

ACKNOWLEDGMENTS

The paper is a part of the Ph.D. thesis study of the first author. He acknowledges the Ayhan Şahenk Agricultural Application and Research Centre for its support during the study.



Author contributions

Conceptualization, AŞ; methodology, AŞ; formal analysis, SHA; investigation, BT, AA, YEŞ, MD; resources, AŞ; data curation, BT, AŞ; writing—original draft preparation, BT; writing—review and editing, BT, AŞ; visualization, BT; supervision, AŞ, MD; project administration, AŞ, MD; funding acquisition, AŞ. All authors have read and agreed to the published version of the manuscript.

Funding

This study was supported by the Scientific Research Projects (BAP) Council of Niğde Ömer Halisdemir University under grant no. TGT 2022/4-BAGEP.

Data availability statement

The research data will be available upon request.

Conflicts of interest

The authors have no competing interests to declare that are relevant to the content of this article.

Appendix

Not applicable.

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