A comparative study on egg cholesterol contents and eggshell protoporphyrin and biliverdin pigments of different poultry species

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ABSTRACT

This study compared the cholesterol levels and shell pigments (protoporphyrin and biliverdin) of chicken (conventional and organic), quail, pheasant, and goose eggs. The material for the study was chicken (organic system - Lohmann Brown and conventional system - HyLine Brown) eggs, quail (*Coturnix coturnix japonica*), goose (local), and pheasant (*Phasianus colchicus*) eggs homogeneously selected with a subjective scoring. High-performance liquid chromatography with photodiode array detection was used to analyze the samples (HPLC-PDA). There were no significant differences in the yolk cholesterol content of eggs between species. Based on mg/g of yolk, different poultry species had comparable amounts of cholesterol. Quail eggshells contained significantly more protoporphyrin (81.92 M/g) than chicken (conventional-organic) and pheasant eggshells (P<0.01), but conventional chicken eggshells contained less protoporphyrin (10.73 M/g) than other species (P<0.01). Biliverdin was found only in the eggshells of quail (2.83 M/g) and pheasant (1.02 M/g) (P<0.01). It was observed that white shelled goose eggs had no detectable pigment. Research is required to elucidate the role of diet, age, stressor, strain, and housing systems on protoporphyrin and biliverdin pigment concentrations and cholesterol in table eggs and breeder eggs production.

Introduction

Besides supplying entire nutrients for normal embryo development, egg offers a great source of nutrients in the daily diets of humans (2). Undoubtedly, a chicken egg is the most widely consumed type of egg (37). It is preferred mainly because of its high nutritional value, better digestibility, low cost and easy accessibility (4). Despite insufficient official data, it was thought that eggs of various other poultry species were served to markets for table consumptions. Besides small-size eggs, like quail eggs, large-size eggs, like goose and duck eggs, are mostly consumed as gourmand materials. Just because of seasonal production, consumption of goose and pheasant eggs is quite limited (37). There has been an increasing interest in quail eggs (22). The chemical composition of eggs of different poultry species is similar to each other, but rational distributions are different (9). Among the egg constituents, cholesterol is a highly significant biological molecule playing a precursor role in cell membrane structure and synthesis of gender and adrenal hormones, bile acids and vitamin D (11). Although egg is related to cardiovascular diseases over high cholesterol levels, *in vivo* and *in vitro* studies revealed a weak correlation between egg cholesterol levels and cardiovascular disease risk (28,31).

Cholesterol intake through the diets is at minimum levels in laying hens. The liver and ovary are the primary organs for cholesterol biosynthesis. Although most yolk cholesterol is synthesized in the liver, transported through the blood in the form of lipoprotein and accumulated in follicles, plasma cholesterol level was not found to be related to egg yolk cholesterol concentration (11). In

poultry species, the egg is the primary means of cholesterol removal. Fecal neutral and acidic sterols represent a secondary means of cholesterol removal (11). Zemkova et al. (42) reported whole egg and egg yolk cholesterol levels respectively as 228.3 mg and 13.3 mg. Kasapidou et al. (18) reported lower yolk cholesterol levels (12.5 g/g) in enriched cases than in litter bed systems (14.1 mg/g). Although it was reported that yolk cholesterol levels were influenced by species, race, age, growing systems and diets (2), it was indicated that cholesterol levels were resistant against the changes in these factors (11).

Eggshell pigmentation was reported as efficient selection-genetic progress in some poultry species (35) and it was also reported that hatching performance was significantly influenced by shell pigmentation (10). Additionally, eggshell pigmentation was also used as an assessment criterion for stress and disease conditions resulting in lighter shell colors in commercial laying hens (29). Although there is a slight or no correlation between brown egg color and nutrient composition (33), brown eggshell color is considered a quality indicator by consumers in several countries (29). The primary shell pigments, protoporphyrin and biliverdin, are the products of heme catabolism; therefore, these molecules could directly be derived from red blood cells, or they can be de nova synthesized in the uterus (34). While protoporphyrin pigment forms red and brown colors on eggshell, green and blue-green eggshell colors are formed by biliverdin (17). Protoporphyrin acts as a prooxidant (16) and increases the breaking resistance of eggshell (5). Biliverdin acts as an essential cytoprotectant and it is a metabolically-produced antioxidant pigment (44). However, the number of studies about the concentration of both pigments in eggshells of chicken and the other poultry species is quite limited.

There is a substantial scientific literature on egg quality, biological value, eggshell pigment synthesis, and deposition (7), but there have been few comparative studies on the egg cholesterol content and eggshell protoporphyrin and biliverdin pigments of chicken, goose, quail, and pheasant. Chicken and quail eggs are primarily used for table egg and breeder, whereas goose and pheasant eggs are occasionally used as table egg, and their eggs vary in color. Therefore, we aim to determine the pigments (biliverdin and protoporphyrin) in chicken, goose, quail, and pheasant. We also measured the egg cholesterol concentration to elucidate the cholesterol level in these species.

Materials and Methods

The material for the study was chicken (organic system - Lohmann Brown and conventional system - HyLine Brown) eggs, quail (*Coturnix coturnix japonica*), goose (local), and pheasant (*Phasianus colchicus*) eggs homogeneously selected with a subjective scoring. Apparent colors of eggshells under daylight: Lohmann Brown and HyLine Brown: brown color; quail: dark brownish white-spotted; goose: white color, and pheasant: dark brown color. Based on the previous result (3) and using α=0.05 and power=90, the projected sample size was approximately 76 for egg yolk cholesterol in total. Thus, we estimated that a sample size of 100 eggs (20 eggs for each poultry species) would be more than adequate to investigate our primary objectives in this study. The eggs used in the experiments were daily eggs and they were not cold-stored. Egg weights were measured with a precise balance (±0.001 g). Chicken (conventional – organic), quail and goose eggs were supplied from a commercial poultry facility in Samsun province and pheasant eggs were supplied from the Pheasant Production Station of the Ministry of Agriculture and Forestry. Homogeneous color and weights were taken into consideration in the selection of the eggs.

***Care and feeding conditions of the facilities from where the eggs were supplied*:** Conventional chickens and quails were housed in cages, pheasants were housed in ground pens (4 m × 5 m) and geese were housed in free-spaces with natural plant cover. Five chickens/m^2 indoor space was provided for organic chickens, and one chicken/4 m^2 outdoor space without plant cover was provided. Conventional chickens were supplied with a ration containing 17% CP and 2800 kcal ME/kg; quails with a ration containing 20% CP and 2900 kcal ME/kg; pheasants with a ration containing 15% CP and 2660 kcal ME/kg. Organic chickens were supplied with an organic poultry feed containing 16% CP and 2627 kcal ME/kg energy and organically grown ground alfalfa hay. Feed and water were supplied ad libitum.

Preparation and extraction of egg yolk samples for cholesterol analysis: Fresh eggs were individually broken and the yolk component was carefully separated. The yolk was washed with distilled water and then rolled on a filter paper to remove adhering white component. The whole yolk was then transferred to a test tube and the yolk membrane was punctured and vortex-mixed for the weighing process. About 2 g yolk sample was weighed into a 50 mL test tube and diluted with 20 mL of distilled water. After vortex-mixing vigorously, 1 mL of diluted sample was transferred into a 15 mL test tube and 1 mL of 95% ethanol was added and the mixture was vortex-mixed vigorously. Then 2 mL of diethyl ether was added into the tube and vortex-mixed vigorously. The former step was repeated with 2 mL of petroleum ether. After holding on for 30 min at ambient temperature, 0.5 ml of the organic phase was pipetted into a new tube and evaporated to

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dryness under a nitrogen stream at 45 °C. The residue was dissolved with 0.5 mL of ethanol, vortex-mixed vigorously and transferred into a 1.5 mL amber vial for HPLC analysis (43).

***HPLC-PDA analysis of egg yolk cholesterol*:** Cholesterol content of the yolk samples was measured by an HPLC system (Prominence LC-20A, Shimadzu, Kyoto, Japan) equipped with a PDA detector (SPD M20A, Shimadzu, Kyoto, Japan) fixed at 208 nm wavelength. A reversed-phase C18 column (Inertsil ODS-3V, 4.6 x 250 mm, 5 µm, GL Science, Tokyo, Japan) was used for separation at an oven temperature of 30 °C. The mobile phase consisted of acetonitrile and 2-propanol (4:1, v/v) isocratically. The flow rate was 0.6 mL/min and the injection volume was 10 µl. The run time was 35 min. A stock solution (1 mg/mL) of cholesterol standard was prepared in ethanol and then eight calibration solutions were adjusted by diluting the stock solutions between the ranges of 0.05-0.40 mg/mL (43).

***Preparation and extraction of eggshell samples for protoporphyrin and biliverdin analysis*:** The eggshell of each egg was broken into small pieces in a mortar and then 100 mg of sample was weighed into a 1.5mL centrifuge tube. An aliquot of 0.5 mL of disodium EDTA solution (100 mg/mL, pH 7.2 using NaOH) was added into the tube, vortex-mixed for 1 min, and the cap was carefully loosened. After reducing effervescence, the tube was centrifuged for 2 min at 15,000 rpm and the supernatant was discarded. These procedures were repeated three times, each allowing the eggshell fragments and EDTA solution to have a contact time of 5 min. Following this step, 1 mL of acetonitrile-acetic acid (4:1, v/v) mixture was added to the tubes and vortex-mixed vigorously for 2 min in 30 s intervals, uncapping the tubes to flow out of CO_2. The supernatant was transferred to a clean tube after centrifugation for 4 min at 15,000 rpm. Finally, the supernatant was filtered through a 0.45 µm PTFE disc filter into a 1.5 mL amber vial, making it ready for HPLC analysis (13).

***HPLC-PDA analysis of eggshell protoporphyrin and biliverdin*:** Protoporphyrin and biliverdin amounts of the eggshell samples were measured by an HPLC system (Prominence LC-20A, Shimadzu, Kyoto, Japan) equipped with a PDA detector (SPD M20A, Shimadzu, Kyoto, Japan) set at 400 and 376 nm wavelengths for protoporphyrin and biliverdin analysis, respectively. Analyses were separated on a Lichrosorb RP-8 column (4 x 250 mm, 5 µm, Merck, Darmstadt, Germany) at oven temperature of 25 °C. The mobile phase A was 100 mM ammonium acetate (pH 5.5 with ortho-phosphoric acid), 2-methoxy ethanol and methanol (45:5:50, v/v) and the mobile phase B was 2-methoxy ethanol and methanol (5:95, v/v). Gradient elution was applied as 100% mobile phase A to 100% mobile phase B over 11 min at a flow rate of 1.4 mL/min and the injection volume was 20 µl. The run time was 15 min. Stock solutions (500 µM) of protoporphyrin and biliverdin standards were prepared and then six calibration solutions (mixed) were adjusted by diluting the stock solutions between the ranges of 0.3-10 µM (19).

Statistical analyses: All data were subjected to analysis of variance (one-way ANOVA) by employing the general linear model procedure of SPSS 21.0 (16). The group means, which are given in Table 1 as mean ± standard deviation, were considered significantly different at the level of $P<0.05$.

Results

Mean values for egg weight, yolk weight and cholesterol levels of chicken (conventional system – organic system), quail, goose and pheasant eggs are provided in Table 1. Naturally, goose eggs had the greatest egg weight and quail eggs had the lowest egg weight. There were no significant differences in egg weights of conventional and organic chickens. The average egg weight was 53.28 g for conventional chicken eggs, 51.04 g for organic chicken eggs, 10.10 g for quail eggs, 128.28 g for goose eggs and 32.72 g for pheasant eggs. There were significant differences in egg yolk weights and total cholesterol levels of investigated species ($P<0.01$) (Table 1, Figure 1). The greatest total cholesterol content was obtained from goose egg (709.45 mg/egg) followed by the conventional chicken egg (218.38 mg/egg), organic chicken egg (200.50 mg/egg), pheasant egg (170.78 mg/egg) and quail egg (55.16 mg/egg). Although total cholesterol level was lower in an organic chicken egg (200.50 mg/egg) than in a conventional chicken egg (218.38 mg/egg), such a difference was not significant. Significant differences were not observed in cholesterol level of 1 g of egg yolk in investigated species.

As can be inferred from Table 1 and Figure 2 and 3, biliverdin and protoporphyrin were not encountered in goose eggshell. The greatest eggshell biliverdin (2.83 µM/g eggshell) and protoporphyrin (81.92 µM/g eggshell) contents were observed in quail eggs ($P<0.01$). As compared to organic chicken eggshells, conventional chicken eggshells had lower ($P<0.01$) protoporphyrin levels (10.73 µM/g eggshell). Pheasant eggshell biliverdin level (1.02 µM/g eggshell) was significantly lower than quail eggshells and pheasant eggshell protoporphyrin level (23.32 µM/g eggshell) was similar with the protoporphyrin level organic chicken eggshell (23.23 µM/g eggshell). It was found that white shelled goose eggs contained no detectable pigment.

Table 1. Egg weight, yolk weight and egg yolk cholesterol, eggshell protoporphyrin and biliverdin pigments values in different poultry species.

Genotype	Egg weight (g)	Yolk weight (g)	Cholesterol (mg/egg)	Cholesterol (mg/g yolk)	Protoporphyrin (µM/g eggshell)	Biliverdin (µM/g eggshell)
C. Chicken	$53.28^{b} \pm 7.71$	$14.66^{b} \pm 2.29$	$218.38^{b} \pm 45.08$	14.96 ± 2.36	$10.73^{c} \pm 10.52$	ND
O. Chicken	$51.04^{b} \pm 2.84$	$12.28^{c} \pm 1.43$	$200.50^{bc} \pm 30.57$	16.96 ± 1.68	$23.23^{b} \pm 14.54$	ND
Quail	$10.10^{d} \pm 1.01$	$3.33^{e} \pm 0.50$	$55.16^{d} \pm 12.79$	16.43 ± 2.06	$81.92^{a} \pm 39.08$	$2.83^{a} \pm 0.98$
Goose	$128.28^{a} \pm 8.38$	$43.10^{a} \pm 6.00$	$709.45^{a} \pm 103.83$	16.61 ± 2.56	ND	ND
Pheasant	$32.72^{c} \pm 2.11$	$10.30^{d} \pm 0.78$	$170.78^{c} \pm 25.53$	16.54 ± 1.96	$23.32^{b} \pm 11.29$	$1.02^{b} \pm 0.60$
P	**	**	**	NS	**	**

C: Conventional; O: Organic; Data are expressed as mean ± standard deviation; $^{a\text{-}e}$: Different superscript letters in a column indicate significant difference; **: P<0.01; ND: not determined; NS: not-significant

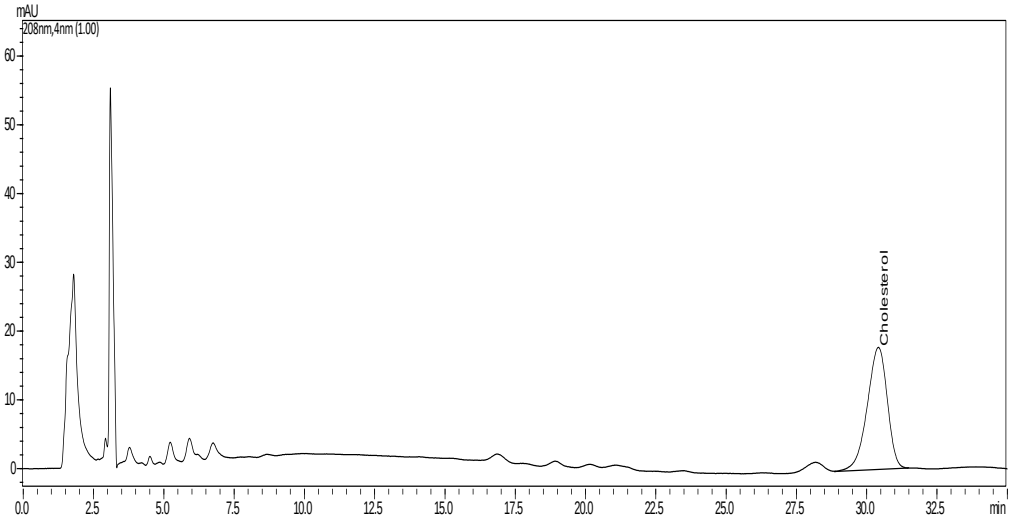


Figure 1. A sample HPLC-PDA chromatogram corresponding to the analysis of egg yolk cholesterol at 208 nm.

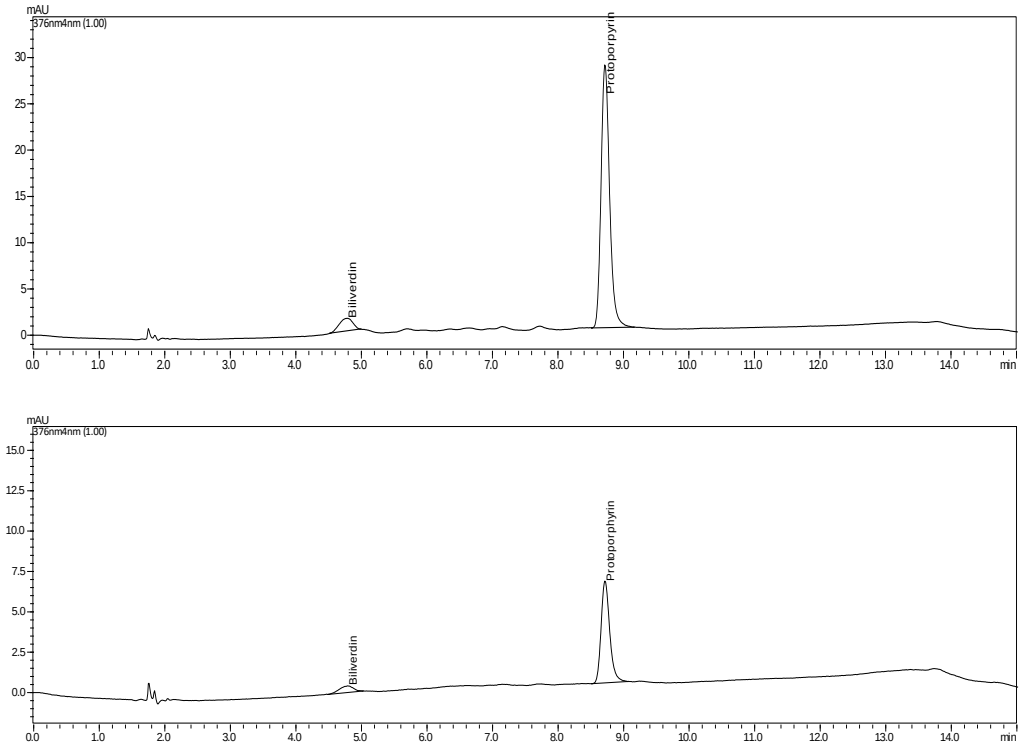


Figure 2. A sample HPLC-PDA chromatogram is corresponding to the analysis of biliverdin in quail eggshell (a) and pheasant eggshell (b) at 376 nm.

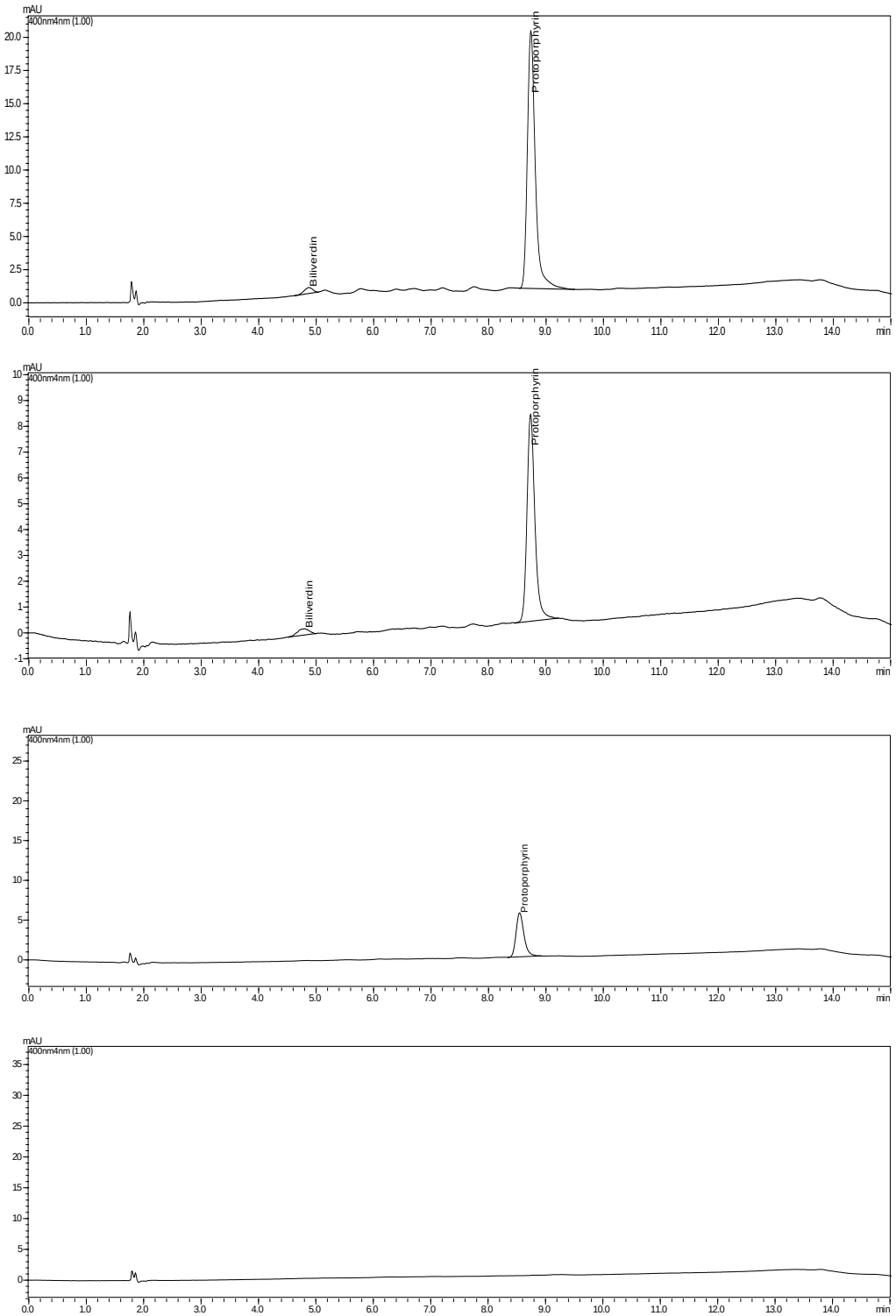


Figure 3. A sample HPLC-PDA chromatogram is corresponding to the analysis of protoporphyrin in quail eggshell (a), pheasant eggshell (b), chicken (organic) eggshell (c) and goose eggshell (d) at 400 nm.

Discussion and Conclusion

As can be inferred from Table 2, there were significant differences in whole egg and egg yolk weights. There is a positive correlation between live weight and egg size of poultry species (25). In previous studies, chicken egg weights were reported as between 60.05 - 67.41 g (25, 36), quail egg weights as between 10.40 - 13.19 g (3, 24), peasant egg weights as between 31.89 - 32.53 g (21, 38) and goose egg weights as between 120 - 195 g (22). In the present study, as compared to conventional chicken eggs, organic chicken eggs had lower egg and egg yolk weights. However, the differences in egg weights were not found to be significant.

In this experiment, the cholesterol content of eggs varies greatly between species when expressed as mg/egg. On an egg basis, it is expected that there will be a difference in cholesterol results. It was found that the cholesterol content of eggs increased with egg weight. These findings are consistent to those of Faitarone et al. (12), who claimed that egg cholesterol levels are positively related to poultry genetics and age, egg weight and yolk weight, and negatively related to lay percentage and dietary protein levels. On the other hand, the cholesterol content of organic chicken eggs (200 mg/g egg) was numerically lower than that of conventional chicken eggs (218.38 mg/g egg). This is thought to be due to the layer diet, which includes herbs from the organic system (23).

The statistical analyses in the current study revealed no significant differences in the yolk cholesterol levels of eggs (mg/g yolk) produced by chickens, quail, goose, and phasant. Adeniyi et al. (1) found a higher average yolk cholesterol content in chicken and quail eggs (33.67 and 46.83 mg/g, respectively) than the current study. Kazmierska et al. (20) observed that chicken, quail, and pheasant eggs had lower yolk cholesterol content (13.91, 7.78, and 6.82 mg/g, respectively) than the current findings. Ukachukwu et al. (39) showed that the overall value for egg yolk cholesterol in quail eggs was 6.79 mg/g, while it was 4.03 mg/g in chicken eggs. Antova et al. (2) and Yalçn et al. (41) reported lower mean values of egg yolk cholesterol (9.95 and 12.52 mg/g, respectively) in white Leghorn and Hyline Brown chickens than the current study. Aziz et al. (4) reported mean values for egg yolk cholesterol in chicken and quail eggs of 7.65 and 16.05 mg/g, respectively. The existing literature mentions various levels of cholesterol in pheasant eggs. Choi et al. (6) reported much higher values (approximately 20 mg/g), while Gugala et al. (6) reported significantly lower (6.8 mg/g of yolk) (15). The difference in egg yolk cholesterol content between the current study and the previous studies could be due to a variety of factors, including laying hen age and diet, production systems, or assay methods (15). Due to limited availability of comparable studies on goose yolk cholesterol, comparing the current study's findings to those in the literature is difficult. According to a previous study (22), the yolk cholesterol content of goose eggs (13.94 mg/g yolk) was lower than that of chicken, quail, and pheasant eggs.

It is of particular importance with respect to sexual signaling and the physiological and mechanical properties of shell pigment (27). The eggshell pigments are influenced by age and genetics (40), and the housing system and nutrition have only a minor impact (8). However, there is much less data on shell pigment concentration. The primary pigment responsible for brown egg coloration is protoporphyrin. Brown eggshells contain traces of biliverdin, which can affect egg color (26). Polin (30) observed that the eggshell glands of brown egg-laying hens have a greater capacity to convert -aminolevulinic acid to porphyrin than other tissues. Protoporphyrin was found to be present in chicken eggs with brown shells in our study. In our study, it was observed that chicken eggs with brown shells were characterized by protoporphyrin. Eggs from organically raised chickens, on the other hand, contained a higher concentration of protoporphyrin than eggs from conventionally raised chickens. In this regard, chickens in the organic system may have produced more protoporphyrin pigments because they were raised in a better environment with appropriate temperature and humidity, as well as under less physiological stress. As a result, if hens are to be raised in a cage system, the conditions required to resolve the problem of poor eggshell color obtained in these systems should be considered. The analysis of eggshell pigment concentration revealed that quail and pheasant eggshells are pigmented with protoporphyrin and, to a lesser extent, biliverdin. Uğurlu et al. (38) found higher biliverdin levels in dark brown, light brown, and green shell colors of pheasant eggs (5.24, 3.72, and 4.72 M/g, respectively) and lower protoporphyrin levels (14.87, 9.44, and 8.68 M/g, respectively) than the current values. Gorchein et al. (14) found protoporphyrin and biliverdin levels in quail eggshells to be 1.66 - 2.17 and 0.25 - 0.40 nmol/mg, respectively. Samiullah and Roberts (32) measured protoporphyrin concentrations in brown eggshells at 33, 50, and 67 weeks as 1.304 10-8, 1.898 10-8, and 1.806 10-8 mM/g, respectively. Changes in eggshell pigment concentrations may reflect physiological conditions such as egg-laying or nesting, but they may also be caused by exogenous (environmental) factors.

In conclusion, non-significant differences in egg yolk cholesterol content were found between species. Quail eggshells contained much higher levels of protoporphyrin than chicken (conventional-organic) and pheasant eggshells, but conventional chicken eggshells contained less protoporphyrin than other species. Only quail and pheasant eggshells contained biliverdin. Research is required to elucidate the role of diet, age, stressor, strain, and housing systems on protoporphyrin and biliverdin pigment concentrations and cholesterol in table eggs and breeder eggs production.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

HM, EA, and AA conceived and planned the experiments. HM, EA, and AA carried out the experiments. HM, EA, and AA planned and carried out the simulations. HM, EA, and AA contributed to sample preparation. HM, EA, and AA contributed to the interpretation of the results. HM took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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