Concentration of egg white antimicrobial and immunomodulatory proteins is related to eggshell pigmentation across traditional chicken breeds

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ABSTRACT Eggshell colour, quality, and biosafety of table eggs are of significant commercial interest. To date, there have been few studies investigating the relationship between eggshell pigmentation and internal egg quality in commercially bred birds. Moreover, the genetic basis and mechanisms behind the effects of extrinsic factors on deposition of antimicrobial compounds in egg white and eggshell pigments are not fully understood. In the present study, we evaluate the effect of chicken breed identity, eggshell pigmentation and the role of extrinsic factors (year and breeder identity) on variability in the concentrations of 2 major egg white antimicrobial proteins (**AMPs**), lysozyme (**LSM**), and ovotransferrin (**OVOTR**), across 23 traditional chicken breeds. We found that chicken breed identity and eggshell pigmentation explained most variability in the concentration of egg white LSM and OVOTR. Year and breeder identity were also significant predictors of egg white LSM and OVOTR variability, and showed selective effects on the deposition of both AMPs in egg white. We also documented a positive correlation between concentration of egg white LSM and eggshell cuticle protoporphyrin in tinted and dark brown eggs, but not in brown, white, and blue eggs. We assume that a combination of both intrinsic genetic and hormonally regulated extrinsic factors is responsible for this relationship and for the variability in egg white AMPs. In this study, we demonstrate the existence of a relationship between eggshell pigmentation and egg white AMPs content in the eggs of traditional chicken breeds that may advertise the egg's antimicrobial potential and biosafety. These findings provide novel insights into the relationship between eggshell pigmentation and egg internal quality and may stimulate the recovery and exploitation of traditional chicken breeds for egg production, where the demands for egg quality and biosafety, in conjunction with animal welfare, are a priority.

Key words: eggshell colour, protoporphyrin IX, albumen, ovotransferrin, lysozyme

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INTRODUCTION

In table egg production, consumers are interested in egg quality, egg safety, and animal welfare. Egg safety plays a particularly important role as, during the process of egg formation and immediately after oviposition, the egg's contents may become contaminated by omnipresent microorganisms in the housing system (De Reu et al., 2008; Chousalkar et al., 2018; Vlckova et al., 2018). Many of these invading microorganisms can be highly pathogenic and pose a health risk for consumers (Cox and Pavic, 2010; Baron and Jan, 2011; Chousalkar and Gole, 2016; Ahmed et al., 2017). However, the egg is equipped with many active compounds that prevent both proliferation of microorganisms on the eggshell and penetration into the egg.

The egg's primary natural defence system is comprised of a range of antimicrobial proteins (**AMPs**) deposited in the outer eggshell and cuticle layer (Nys et al., 2004; Wellman-Labadie et al., 2008a; Gautron et al., 2011; Miksik et al., 2014). The cuticle plays an essential role in reducing microorganisms on the eggshell, and thus protects the egg's contents against potentially invading microbes (Bain et al., 2013, 2019; Samiullah and Roberts, 2014; D'Alba et al., 2017; Chen et al., 2019). Previous studies have noted, however, that cuticle deposition can be affected by the eggshell's colour and the hen's age (Kulshreshtha et al., 2018) or genotype (Ketta and Tumova, 2018). A second, but equally important, antimicrobial defence system for protecting the egg's interior is a chemical barrier composed of miscellaneous egg white AMPs (Wellman-Labadie et al., 2008b,c; Sun et al., 2017; Svobodová et al., 2019). Among these, lysozyme (**LSM**), ovotransferrin (**OVOTR**), ovomucoid, ovoinhibitor, and ovostatin appear to be the most important (Stevens, 1991). OVOTR is one of the most abundant egg white proteins and

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plays a crucial physiological and immunomodulatory role during embryogenesis (Giansanti et al., 2012). Owing to its ability to chelate iron, an essential compound for bacterial growth (Baron and Jan, 2011; Baron et al., 2014), OVOTR acts as a bacteriostatic, and may also act as a bactericide by damaging the bacterial cytoplasmic membrane (Ibrahim et al., 2000). Furthermore, OVOTR derived peptides have been shown to display antiviral and antifungal properties (Giansanti et al., 2005). As such, OVOTR is an essential multi-functional protein with strong antimicrobial and immunomodulatory potential for avian/chicken embryos. LSM displays bacteriostatic activity (Masschalck and Michiels, 2003) and is the main protein showing direct lytic antimicrobial activity through cell wall hydrolysis of Gram-positive bacteria (Ibrahim et al., 1994, 2001; Wellman-Labadie et al., 2008b).

In addition to the 2 main egg protection systems introduced above, eggshell pigmentation has also been shown to have antimicrobial properties. In laying hens, eggshell pigmentation may be blue or range from white to dark brown. The blue eggshell colour is due to deposition of biliverdin and zinc biliverdin pigments, while the more abundant brown colours are due to differing intensities of the pigment protoporphyrin IX (**PROTO IX**) (Miksik et al., 1996). Ishikawa et al., (2010) noted that both of these pigments are involved in light-dependent inactivation and reduction of microorganisms on the eggshell. Although a few studies have found an association between pigmentation and nesting ecology that could predict intensity of microbial pressure on a clutch (Maurer et al., 2011; Cassey et al., 2012), the only experimental study on free-living birds found no support for this pigment-dependent antimicrobial hypothesis (Dearborn et al., 2017).

Both AMP concentration and pigmentation can vary substantially in laying hens and across chicken breeds, with LSM and OVOTR concentrations varying with physiological status and age of the laying hen (Sellier et al., 2007; Vlckova et al., 2019) or in hens that have previously experienced intensive infection (Bedrani et al., 2013a). Moreover, Bilkova et al., (2018) reported breed-specific variability in egg white proteomic profiles due to variable immunological adaptation, reflecting their original need to fight pathogens during the domestication process. Thus, even if deposition of egg white AMPs is breed-specific and genetically determined (Bilkova et al., 2018; Zhang et al., 2019), it is also strongly affected by hormonally mediated external factors (Palmiter et al., 1981). Likewise, pigmentation intensity may reflect hen/flock ageing (Odabasi et al., 2007; Samiullah et al., 2017; Bi et al., 2018), stress and laying hen condition status (Mertens et al., 2010; Duval et al., 2013), as well as external and internal egg quality (Joseph et al., 1999; Butler and McGraw, 2013; Samiullah et al., 2017). While deposition of brown and blue pigment is strongly genetically determined (Zheng et al., 2014; Li et al., 2016; Wang et al., 2017; Bai et al., 2019), being caused by retrovirus insertion into the chicken genome in blue-shelled breeds (Wang et al., 2013), the intensity of pigmentation may also be controlled by extrinsic factors regulated by sex steroid hormones and glucocorticoids (Soh et al., 1993; Soh and Koga, 1994). Taking into account that a hen's genetic and hormonal background controls deposition of both eggshell pigment and egg white AMPs during ovogenesis (Palmiter et al., 1981; Soh et al., 1993; Soh and Koga, 1994), we predict that there may exists an association between pigmentation and concentration of the main egg white AMPs. To date, however, very few studies have focused on the relationship between concentration of egg shell pigment and AMPs (Nowaczewski et al., 2013; Butler and Waite, 2016). Furthermore, there is a lack of studies investigating associations between pigmentation and the antimicrobial and immunomodulatory capacity of egg white across traditional chicken breeds, which represent a neglected source of genetic variability linked with different selective pressures during the domestication process (Rubin et al., 2010).

In this study, we investigate the role of chicken breed identity, eggshell pigmentation, and the extrinsic factors (year and breeder identity) on variability in the concentrations of two main egg white AMPs (LSM and OVOTR), across 23 traditional chicken breeds. Moreover, we assess the association between concentration of eggshell cuticle PROTO IX and LSM/OVOTR concentrations. The findings of this study provide a solid background for the association between eggshell colour and egg internal quality, and between intrinsic and extrinsic factors affecting the deposition of egg white antimicrobial and immunomodulatory compounds across traditional chicken breeds. These factors are of great importance in breeding programmes, particularly as regards selection for optimal genotypes producing biosecure eggs with enhanced antimicrobial potential.

MATERIAL AND METHODS

Chicken Breeds and Egg Collection

In total, we processed 380 freshly laid eggs of 23 traditional chicken breeds from 41 non-commercial breeders in the Czech Republic (Supplementary Table S1). The eggs were collected during spring 2013 to 2015 over 4 consecutive days and processed immediately after delivery (i.e., the eggs were no more than 6-days-old eggs when processed). Each egg was assigned to one of five colour categories based on the intensity of blue/brown pigmentation, i.e., blue, white, tinted, brown, and dark brown (see Supplementary Table S1 for details). For each colour category, we analysed mean concentration of eggshell cuticle PROTO IX (see Biochemical analysis below) in the subset represented by selected chicken breed (Supplementary Table S2). Eggshell cuticle PROTO IX concentration varied significantly between colour categories and corresponded with brown

eggshell pigmentation intensity (Supplementary Figure S1).

Egg Processing

The length and width of each egg was measured to the nearest 0.01 mm with Quatros® QS15506 digital callipers (Lublin, Poland) and the egg volume calculated according to the method of Narushin (2005). The eggshell cuticle was then extracted from each egg following the method of Kennedy and Vevers (1976). Briefly, each egg was put into a 80 × 120 mm plastic bag containing 40 ml of 5% EDTA (Sigma-Aldrich GmbH, Steinheim, Germany) and 10 mM 2-mercaptoethanol (PENTA, Prague, Czech Republic) solution buffered to pH = 7.4 with NaOH (PENTA, Prague, Czech Republic) for a period of 1 h. The egg was then removed, placed onto a Petri dish (Deltalab, Barcelona, Spain) and the cuticle gently scraped away using a plastic scalpel and washing bottle filled with ddH_2O. The released cuticle in double-distilled water (ddH_2O) was then dialysed using the Pur-A-Lyzer™ Mega Dialysis Kit (Sigma-Aldrich GmbH, Steinheim, Germany) and lyophilised using Alpha 1–2 LD plus (Martin Christ GmbH, Osterode am Harz, Germany). The lyophilised cuticle was then weighed to the nearest 0.001 g using an ABT 120–5DM analytical laboratory scale (Kern & Sohn GmbH, Balingen, Germany) and stored at -80°C until PROTO IX analysis. After this procedure, each egg was washed with ddH_2O to remove any residue of EDTA/Mercaptoethanol solution, dried, manually cracked and the yolk and egg white gently separated. The egg white was transferred to a 50 ml cryotube (Nunc™, Thermo-Fisher Scientific, Waltham, MA, USA) and stored at -80°C until LSM and OVOTR analysis.

The experimental procedure was conducted with the approval, and under the supervision of, the Ethical Committee of the Faculty of Science, Charles University, Prague (permit no. 13,060/2014-MZE-17,214).

Biochemical Analysis

Concentration of Egg White Lysozyme Egg white LSM concentration was measured using the agar well-diffusion assay (Osserman and Lawlor, 1966). Briefly, we prepared a solution of Britton–Robinson buffer (pH = 7.0) containing 0.492 g boric acid (Alchimica, Prague, Czech Republic), 0.782 g phosphoric acid (98%; Lach-Ner, Neratovice, Czech Republic), 0.480 g acetic acid (Lach-Ner, Neratovice, Czech Republic), and 0.840 g NaOH (Alchimica, Prague, Czech Republic) dissolved in 305 ml of distilled water. Following this, 50 mg of lyophilised Micrococcus lysodeikticus (ATTC 4698, M3770, Sigma-Aldrich GmbH, Steinheim, Germany) was re-suspended in 10 ml of Britton-Robinson buffer and added to a 60°C solution of 1% agar (Alchimica, Prague, Czech Republic) re-suspended in 100 ml of Britton-Robinson buffer. The agar was poured into Petri dishes and left for 30 minutes to solidify, after which 3-mm diameter holes were punched into the agar using a core borer. Egg white samples were thawed and homogenised with a magnetic stirrer (IKA RH Digital) at 1800 rpm for 30 min, after which 10 μl of the homogenised sample was transferred into duplicate holes in the agar plates using GENO-DNA S pipette tips for viscous liquids (CS960 9,405,120, Thermo-Fisher Scientific, MA, Waltham, USA). Standard LSM solutions (10 μl) of known concentration (20, 15, 7, 4, 2, 0.5 mg/ml) were prepared by diluting lyophilised egg white LSM (HEWL, L6876, Sigma–Aldrich, St. Louis, MO, USA) in Britton–Robinson buffer and these were added to the holes in the agar plates. The plates were then incubated for 24 h at 21°C and 50 to 60% humidity. Photographs of the plates showing the clearance zones around each hole were taken in standard position using a Canon EOS 450D camera (Canon cameras, Japan) with a 50 mm macro objective (F2.8). The clearance zone diameter was analysed using ImageJ 1.42q software (Schneider et al., 2012). LSM concentration (mg/ml) for each egg white sample was interpolated from a calibration curve using GraphPad Prism v. 6.00 for Windows (GraphPad Software, San Diego California USA).

Concentration of Egg White Ovotransferrin OVOTR concentration was measured using a modified version of the iron-binding activity assay (Yamanishi et al., 2002), described in detail in Horrocks et al., (2011). Briefly, 25 μl of homogenised egg white sample (see above) was transferred into a 96-well microplate (BRAND® microplate, pureGrade, flat-transparent, Sigma–Aldrich, St. Louis, MO, USA) in quadruplicate. In order to create a calibration curve, we prepared a stock OVOTR solution containing 40 mg of OVOTR (Conalbumin, C0755, Sigma–Aldrich, St. Louis, MO, USA) dissolved in "reagent 1" containing 200 ml ddH_2O, 7.3 g Tris, 6.4 g Na_2CO_3, 0.84 g Triton-X (Sigma–Aldrich, St. Louis, MO, USA). Duplicate 25 μl samples of OVOTR standard solutions ranging from 30 mg/ml to 0.1 mg/ml concentration were placed in the bottom row of wells in each plate. Next, 120 μl of "reagent 2" containing 150 ml of "reagent 1" and 600 μl of iron standard solution (VWR International, Lutterworth, England) was added to each well, following which the plate was shaken for 10 s. "Pre-reads" were obtained using a TECAN Infinite1200 PRO UV/Vis microplate reader (Tecan Group Ltd., Switzerland) at absorbance wavelengths of 570 nm and 660 nm. After incubation at 37°C for 5 min, we added 25 μl of ascorbic acid solution (100 ml ddH_2O, 0.49 g FerroZine™, 0.6 g Tris, 0.574 g ascorbic acid) to each well and placed it back in the incubator for 5 min at 37°C. Finally, 100 μl of "reagent 3" (200 ml ddH2O, 25.2 g citric acid, 0.38 g thiourea) was added and the absorbance (570 nm and 660 nm wavelength) was recorded immediately (t = 0) and after a further 6 min incubation at 37°C (t = 7). The absorbance values were

corrected for initial values of well-specific "pre-read" absorbance and normalised using reference absorbance values at 660 nm wavelength. The difference between values measured at t = 0 and t = 7 were used for calculation of OVOTR concentration by interpolation of the standard curve in GraphPad Prism 6.

Concentration of Protoporphyrin IX in the Eggshell Cuticle PROTO IX was assessed on a subset of 47 cuticles from eggs of 18 chicken breeds (see Supplementary Table S2) in the form of dimethyl ester, using a procedure based on that of Miksik et al., (1996) for determination of porphyrins in eggshell. Samples were extracted (and esterified) from the eggshells by placing them in 5 ml absolute methanol (LiChrosolv, gradient grade for chromatography, Merck, Darmstadt, Germany) containing 5% concentrated H_2SO_4, in the dark at room temperature and under an N_2 atmosphere for2 d. The extracts were then filtrated, a 4 ml chloroform (Merck, Darmstadt, Germany; chloroform GR, ISO) and 4 ml distilled water solution added and then shaken. The lower chloroform phase was collected and the upper aqueous phase was again extracted with chloroform, the 2 chloroform phases from both extractions being pooled. These phases were washed with 2 ml 10% NaCl, followed by distilled water, until the washings were neutral. The extracts were then evaporated to dryness and reconstituted in 0.5 ml chloroform with 5,10,15,20-tetra(4-pyridyl)-21H,23H-porphine internal standard (Sigma-Aldrich, St. Louis, MO, USA; 0.01 mg/ml). Standards for quantification (protoporphyrin IX, MP Biomedicals, LLC, Eschwege, Germany) were treated using the same procedure.

PROTO IX pigment was determined and quantified by reverse-phase high-performance liquid chromatography (**HPLC**) using the Agilent 1100 LC system (Agilent, Palo Alto, CA, USA), which consists of a degasser, a binary pump, an autosampler, a thermostatted column compartment and a diode-array detector. The HPLC was also coupled to an Agilent LC-MSD Trap XCT-Ultra ion-trap mass spectrometer (ion-trap MS; Agilent, Palo Alto, CA, USA). Chromatographic separation was carried out in a Gemini 5 u C18 110A column (250 × 2.0 mm I.D., Phenomenex, Torrence, CA, USA). A 10 μl sample was injected into the column and eluted with a linear gradient (A = water with 0.1% formic acid, B = acetonitrile with 0.085% formic acid), a flow rate of 0.35 ml/min and a temperature of 55°C. The gradient started at A/B 80:20, reached 10:90 rations after 15 min and reached 100% B after 5 min, the elution being isocratic for the next 10 min. Elution was monitored by absorbance at 375 and 410 nm. Atmospheric pressure ionisation-electrospray ionisation positive mode ion-trap MS in multiple reaction monitoring mode was used when precursor ions were 619 (internal standard) and 591 (PROTO IX), with operating conditions set at drying gas N_2 at 11 l/min, drying gas temperature 350°C, and nebuliser pressure 30 psi (207 kPa).

Table 1. GLM results evaluating the effect of eggshell pigmentation, breed, egg volume, breeder identity, and year of egg collection on the concentration of egg white lysozyme in eggs (N = 380) from traditional chicken breeds.

Explanatory variable	df	Sum sq.	Mean sq.	F	*P* value
Eggshell pigmentation	4	0.442	0.110	17.891	*<0.001
Chicken breed	18	0.881	0.050	7.929	*<0.001
Egg volume	1	0.005	0.005	0.856	0.355
Year	2	0.214	0.107	17.290	*<0.001
Breeder	28	0.394	0.014	2.281	*<0.001

Statistics for particular explanatory variables corresponded to the minimum adequate model when each non-significant term was removed from the model using a backward stepwise procedure.

GLM, General Linear Model.

*Marks significant effects of explanatory variables ($\alpha = 0.05$).

For high concentrations of PROTO IX (15,000–15 ng/ml), absorbance was set at 410 nm. The calibration curve over this range was linear with a regression coefficient of $R^2 = 0.9947$, the limit of detection being 0.15 ng/ml.

Statistical Analysis

Egg white LSM and OVOTR concentrations were not correlated (Pearson's product moment correlation: t = 3.360, $P < 0.12$; $R^2 = 0.03$); hence, we used 2 separate General Linear Models (**GLMs**) with Gaussian error distribution to analyse the effect of pigmentation, egg volume, breed, breeder identity, and year of collection on LSM and OVOTR variability. Pigmentation, egg volume, breed, breeder identity and year of collection were included as explanatory variables in each GLM. Data for mean LSM and OVOTR concentration were log-transformed to achieve normality.

As we sampled the eggs for each chicken breed from multiple breeders and over three consecutive years, year of collection was a strong predictor of LSM and OVOTR concentration in the GLMs ($P < 0.001$ and 0.01, respectively; Tables 1 and 2). Similarly, breeder identity strongly affected LSM concentration ($P < 0.001$, Table 1). To account for such statistical non-independence in our data, we used 2 separate Generalised Linear Mixed Models (**GLMMs**) to test for the effect of breed, pigmentation and egg volume on LSM and OVOTR concentration, with year of collection and breeder identity included as random effects in each GLMM. To evaluate the percentage of variability explained by the random and fixed effect terms, we used the component of variability function "VarrCorr" in the R package *lme4* (Bates et al., 2015).

Factors associated with variation in cuticle PROTO IX concentration were assessed on a subset of 47 cuticles from 18 chicken breeds using separate GLMs. Log-transformed cuticle PROTO IX concentration was set as a response variable and pigmentation nested within breed, egg volume, LSM and OVOTR concentration and their two-way interactions, with pigmentation set as an explanatory variable.

Table 2. GLMM results explaining the effect of eggshell pigmentation, breed, and egg volume on the concentration of egg white lysozyme and ovotransferrin in eggs of traditional chicken breeds.

		Egg white LSM			Egg white OVOTR	
Explanatory variable	df	χ^2	*P* value	df	χ^2	*P* value
Eggshell pigmentation	4	13.812	0.008	4	18.475	*≪0.001
Chicken breed	18	30.000	0.037	18	31.850	*0.023
Egg volume	1	0.137	0.712	1	3.303	0.069

Breeder identity and year of egg collection were set as random effects in GLMM.
GLMM, Generalised Linear Mixed Model; LSM, lysozyme; OVOTR, ovotransferrin.
*Marks significant effects of explanatory variables ($\alpha = 0.05$).

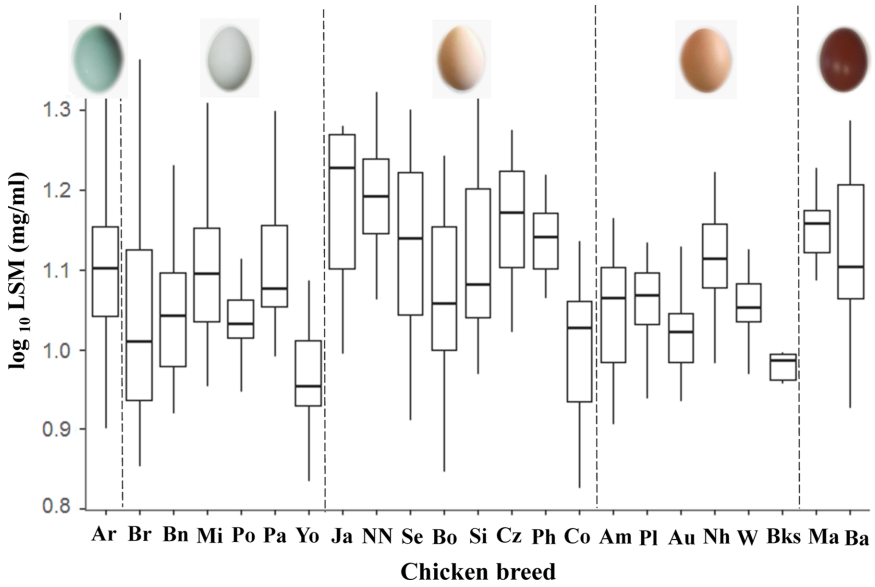

Figure 1. Variability in egg white lysozyme (LSM) concentration ($\log_{10}$ mg/ml) across traditional chicken breeds diverging in eggshell pigmentation (**Ar**—Araucana, **Br**—Brabanter, **Bn**—Rosecomb Bantam, **Mi**—Minorca, **Po**—Poland, **Pa**—Padua, **Yo**—Yokohama, **Ja**—Japanese Bantam, **NN**—Transylvanian Naked Neck, **Se**—Sebright Bantam, **Bo**—Booted Bantam, **Si**—Silke, **Cz**—Czech, **Ph**—Phoenix, **Co**—Cochin, **Am**—Amrock, **Pl**—Plymoth Rock, **Au**—Australorp, **Nh**—New Hampshire, **W**—Wyandotte, **Bks**—Ko Shamo Bantam, **Ma**—Marans, **Ba**—Barnevelder. Vertical dashed lines define what breeds belong to the particular eggshell pigmentation category.

Backwards-stepwise elimination of non-significant terms was performed to select the best minimal adequate model (Crawley, 2007). Significant effects between categorical variables were estimated using *a posteriori* comparison tests and expressed as means ± 95% CI. All analyses and graphics were undertaken using RStudio software v.1.1.463 (RStudio Team 2015), using the *lme4* (Bates et al., 2015), *multcomp* (Hothorn et al., 2008) and *ggplot2* (Wickham, 2016) packages.

RESULTS

Factors Responsible for Variability in Egg White Lysozyme and Ovotransferrin Concentration

There was a significant relationship between pigmentation, breed, year of collection and breeder identity and variability in concentration of egg white LSM in traditional chicken breeds (all $P < 0.001$; Table 1). Concentration of egg white LSM varied greatly across chicken breeds with differently pigmented eggs (Figure 1), with the effect of breed and pigmentation on LSM concentration remaining strongly significant after controlling for the effect of breeder identity and year of collection (Table 2). There was no relationship between egg volume and LSM variability (Tables 1 and 2). "Dark brown" eggs, followed by "tinted" eggs, had significantly higher concentrations of LSM than "white" and "brown" eggs, but no significant difference between "blue" and "dark brown" or "tinted" eggs (Figure 2).

Similar outcomes were documented for egg white OVOTR, with concentration varying significantly across traditional chicken breeds ($P < 0.001$; Table 3, Figure 3), between differently pigmented eggs ($P < 0.001$; Table 3, Figure 4) and with year of collection ($P < 0.001$) and egg volume (P = 0.022), with no significant effect of breeder identity (Table 3).

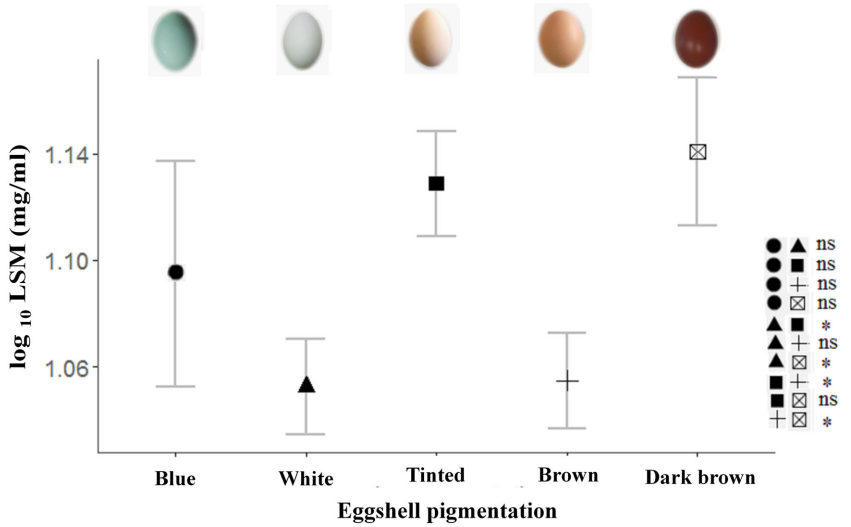

Figure 2. Differences (mean ± 95% confidence intervals) in egg white lysozyme (LSM) concentration ($\log_{10}$ mg/ml) in differently pigmented chicken eggs. Significant differences based on *a posteriori* comparison tests between eggshell colour categories are denoted in the legend. Asterisks represent significant differences at $P < 0.05$ and "ns" indicates no statistical difference.

Table 3. GLM results evaluating the effect of eggshell pigmentation, breed, egg volume, breeder identity, and year of egg collection on the concentration of egg white ovotransferrin in eggs (N = 256) of traditional chicken breeds.

Explanatory variable	df	Sum sq.	Mean sq.	F	*P* value
Eggshell pigmentation	4	3.480	0.870	11.011	*<0.001
Chicken breed	18	4.323	0.240	3.041	*<0.001
Egg volume	1	0.418	0.418	5.298	*0.022
Year	2	1.254	0.627	7.935	*<0.001
Breeder	27	2.241	0.083	1.029	0.431

Statistics for particular explanatory variables corresponded to the minimum adequate model when each non-significant term was removed from the model using a backward stepwise procedure.

GLM, General Linear Model.

*Marks significant effects of explanatory variables ($\alpha = 0.05$).

After controlling for the effect of year of collection and breeder identity, only the pigmentation and breed remained significant (Table 2). Concentration of OVOTR was significantly higher in "tinted" eggs than "blue", "white" and "brown" eggs (Figure 4), whereas there was no significant difference between "tinted" and "dark brown" eggs (Figure 4). Lowest OVOTR concentrations were found in "blue" eggs, followed by "brown" eggs, while "white" eggs had OVOTR concentrations comparable with "dark brown" eggs.

Analysis of variance components revealed that the main effect of breed-associated pigmentation explained 52 and 66% of overall variability in LSM and OVOTR concentration, respectively, while year of collection explained 19 and 29% of overall variability in LSM and OVOTR, respectively. In comparison, we found a strong difference in variability explained by the effect of breeder, with breeder identity explaining 29% of overall variability in LSM, but only 5% of overall variability in OVOTR, which corresponds with the observed non-significant effect of breeder identity on OVOTR concentration (Table 3).

Factors Related to Variability in Eggshell Cuticle Protoporphyrin IX Concentration

Variability in cuticle PROTO IX concentration was significantly affected by eggshell pigmentation (Table 4 and Figure S1) and there was a significant interactive effect of eggshell pigmentation and egg white LSM concentration (Table 4). While LSM concentration increased with increasing cuticle PROTO IX concentration in "tinted" ($t = 2.914$, $P < 0.01$) and "dark brown" eggs ($t = 2.681$, $P = 0.01$), there was no relationship with cuticle PROTO IX in "blue" ($P = 0.221$), "white" ($P = 0.143$) or "brown" ($P = 0.367$) eggs (Figure 5).

DISCUSSION

In this study, we found that egg white LSM and OVOTR concentration varied significantly with chicken breed identity. This is in accordance with the study of Bilkova et al., (2018), who documented breed-specific egg white protein abundance in 5 traditional chicken breeds resulting from differing selective pressures and evolutionary histories during the domestication processes of particular chicken breeds. There is extensive support for a genetic basis to secretion of the main egg white proteins, including LSM and OVOTR. These studies have documented genetic polymorphism in the genes encoding both AMPs in commercially breeding birds, resulting in differing antimicrobial potentials for LSM and OVOTR (Myint et al., 2012a,b; Kinoshita et al., 2016). Moreover, genome-wide analysis has revealed positive selective signatures in the genome of breeds subjected to artificial selection for the production of meat and eggs (Zhang et al., 2017). We analysed numerous traditional chicken breeds selected for different phenotypic traits from distinct regions of the world (see Supplementary Table S1). We observed some

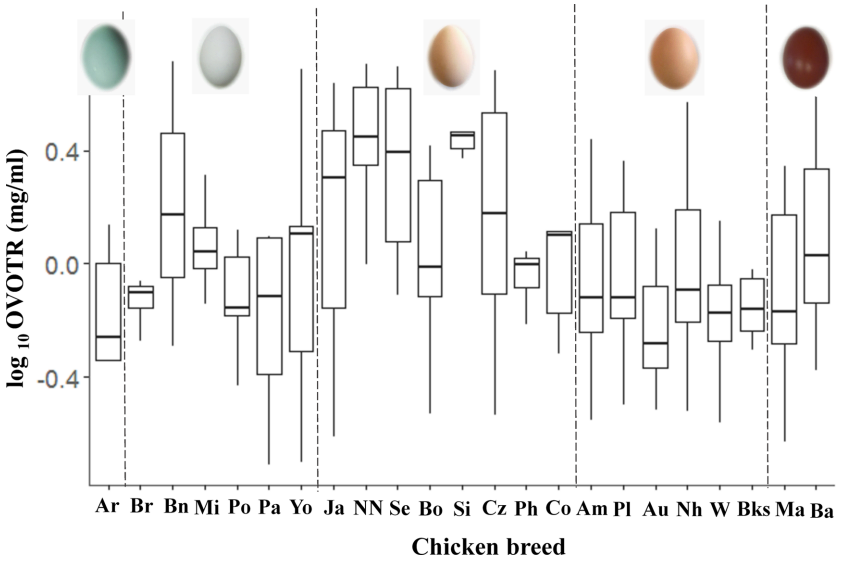

Figure 3. Variability in egg white ovotransferrin (OVOTR) concentration ($\log_{10}$ mg/ml) across traditional chicken breeds diverging in eggshell pigmentation (**Ar**—Araucana, **Br**—Brabanter, **Bn**—Rosecomb Bantam, **Mi**—Minorca, **Po**—Poland, **Pa**—Padua, **Yo**—Yokohama, **Ja**—Japanese Bantam, **NN**—Transylvanian Naked Neck, **Se**—Sebright Bantam, **Bo**—Booted Bantam, **Si**—Silke, **Cz**—Czech, **Ph**—Phoenix, **Co**—Cochin, **Am**—Amrock, **Pl**—Plymoth Rock, **Au**—Australorp, **Nh**—New Hampshire, **W**—Wyandotte, **Bks**—Ko Shamo Bantam, **Ma**—Marans, **Ba**—Barnevelder. Vertical dashed lines define which breeds belong to a particular eggshell pigmentation category.

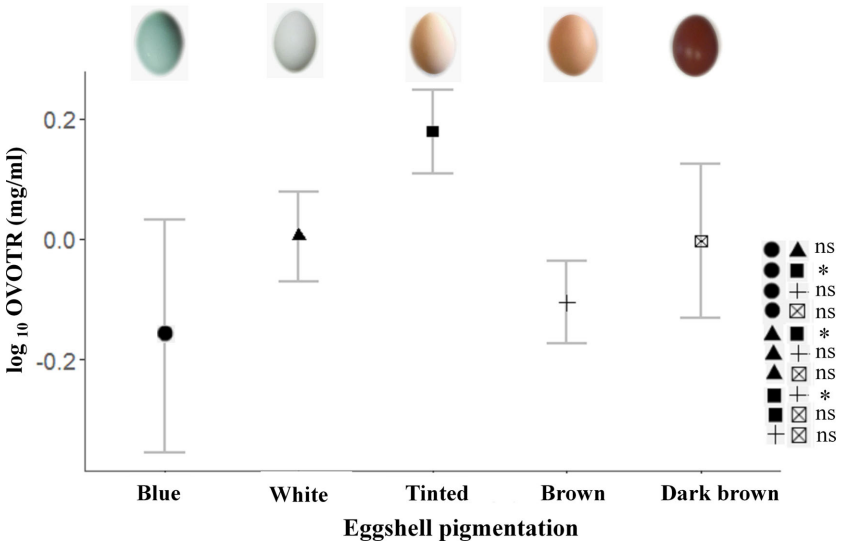

Figure 4. Difference (mean ± 95% confidence intervals) in egg white ovotransferrin (OVOTR) concentration ($\log_{10}$ mg/ml) in differently pigmented chicken eggs. Significant differences based on *a posteriori* comparison tests between eggshell colour categories are denoted in the legend. Asterisks represent significant differences at $P < 0.05$ and "ns" indicates no statistical difference.

trend of breeds primary selected for ornamental purposes (i.e., most of breeds having "white" and "tinted" eggshells) to have higher egg white OVOTR concentration compared to breeds selected primary for egg laying or dual-purposes and producing "blue", "brown" and "dark-brown" eggs. This may support primarily immunomodulatory function of egg white OVOTR which may protect and favour developing embryos of breeds where health and good physical condition of chicks are primary characteristics. Besides, it is highly probable that the different evolutionary histories of particular breeds will play an important role in genetic variability and polymorphism of genes coding secretion of egg white AMPs, accounting for the observed breed-specific concentrations of LSM and OVOTR.

Similarly, eggshell pigmentation significantly explained 52 and 66% of overall variability in egg white LSM and OVOTR concentration, respectively, across traditional chicken breeds, with "tinted" and "dark brown" eggs having significantly higher LSM concentrations than "white" and "brown" eggs. We observed an identical pattern as regards egg white OVOTR, except that "tinted" eggs had the highest OVOTR concentration, and significantly higher OVOTR concentrations than "brown", "white" and "blue" eggs, the latter having the lowest OVOTR concentration. Taken together,

Table 4. GLM results on the relationship between eggshell cuticle protoporphyrin IX concentration in eggs (N = 50) of 19 traditional chicken breeds and egg white lysozyme and ovotransferrin concentration, eggshell pigmentation, egg volume, and their interactions.

Explanatory variable	df	Sum sq.	Mean sq.	F	*P* value
Eggshell pigmentation	4	15.159	3.790	11.070	*<0.001
LSM concentration	1	0.146	0.146	0.427	0.521
OVOTR concentration	1	0.397	0.397	1.233	0.284
Egg volume	1	0.041	0.041	0.130	0.724
Pigmentation:Chicken breed	14	7.221	0.516	1.507	0.192
Pigmentation:LSM concentration	4	5.264	1.316	3.844	*0.017
Pigmentation:OVOTR concentration	4	0.673	0.168	0.523	0.720

Statistics for particular explanatory variables corresponded to the minimum adequate model when each non-significant term was removed from the model using a backward stepwise procedure.

GLM, General Linear Model; LSM, lysozyme; OVOTR, ovotransferrin.

*Marks significant effects of explanatory variables or their interactions ($\alpha = 0.05$).

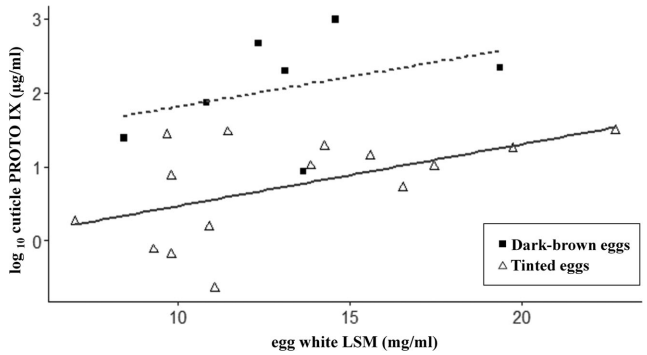

Figure 5. Relationship between concentration of eggshell cuticle protoporphyrin (PROTO IX) and concentration of egg white lysozyme (LSM) in tinted eggs (N = 15) including Transylvanian Naked Neck, Sebright Bantam, Booted Bantam, Silke, Czech, Phoenix and Cochin breeds and dark-brown eggs (N = 7) including Marans and Barnevelder breeds.

traditional chicken breeds producing "tinted" eggs had the highest concentrations of essential AMPs, LSM and OVOTR. This partially corresponds with the study of Kozuszek et al., (2009), who documented higher LSM concentrations in blue and light-brown pheasant eggs compared with olive and dark-brown eggs. To date, there has been only one similar study on commercial chicken breeds, which documented no relationship between eggshell colour and egg internal quality (Yang et al., 2009). On the other hand, several studies on free-living birds have shown an association between the intensity of protoporphyrin- and/or biliverdin-based pigmentation and egg internal quality (Hargitai et al., 2016; Ugurlu et al., 2017; Hargitai et al., 2018; Soler et al., 2018). It would appear, therefore, that our findings are in agreement with the studies on free-living birds; however, further investigation is needed on the association between eggshell pigmentation and egg internal quality in commercial breeding birds.

In addition to a high likelihood of intrinsic genetic factors being responsible for the observed variation in LSM and OVOTR in differently pigmented eggs of traditional chicken breeds, we also documented the effect of environmental (year of collection) and breeding condition (breeder identity) factors. While year of collection explained 19% of overall variability in LSM, the effect was higher as regards OVOTR, explaining 29% of overall variability. The role of environmental factors, such as temperature (Horrocks et al., 2014), humidity (Wellman-Labadie et al., 2008a) and intensity of microbial pressure (Bedrani et al., 2013a,b) has previously been documented as affecting the concentration of egg white AMPs in free-living birds. Hence, it is highly likely that identical mechanisms mediate the effects of these environmental factors on changes in egg white AMP concentration in traditional chicken breeds. We also observed an effect of breeding condition (i.e., breeder identity) on deposition of egg white AMPs. This is in accordance with previous studies documenting the role of diet (Carvalho et al., 2018) and housing condition on egg white amino acid profiles and protein characteristics, including Haugh unit (Samiullah et al., 2017). Moreover, He et al., (2017) noted that the considerable effect of diet on the potential of magnum tubular cells to secrete egg white proteins was hormonally regulated in commercial chicken lines. In our study, however, we revealed that breeding conditions affect deposition of LSM and OVOTR differently. While breeding conditions explained 29% of overall variability in LSM concentration, its effect on OVOTR concentration was only minor, explaining just 5% of overall variability. These findings suggest that the role of breeding condition, include housing and diet quality, on deposition of egg white AMPs is selective and affects deposition of LSM and OVOTR differently, with LSM deposition being more sensitive to these factors and OVOTR deposition almost unaffected. These findings were also supported by the fact that LSM and OVOTR concentrations were not correlated in egg white, suggesting different and independent regulatory mechanisms mediating the effect of breeding and environmental conditions on LSM and OVOTR deposition in traditional chicken breeds.

Finally, we documented a positive correlation between concentration of egg white LSM and cuticle PROTO IX concentration, but only in "tinted" and "dark brown" eggs. The most probable explanation for

this association may be that very similar hormonal pathways, with sex steroid hormones and glucocorticoids playing a dominant role, may regulate secretions of both PROTO IX in the eggshell gland (Soh et al., 1993; Soh and Koga, 1994) and AMPs in the hen's oviduct (Palmiter et al., 1981). This may lead to an increase/decrease in the secretion of either compound or their inter-correlation. Moreover, this hormonal explanation may be supported by the absence of any relationship between concentrations of egg white OVOTR and cuticle PROTO IX. Based on our findings, OVOTR deposition is less sensitive to extrinsic factors, such as breeding condition, and thus is probably subject to intrinsic genetic factors rather than hormonal regulatory factors, which may help explain the lack of egg white OVOTR and cuticle PROTO IX relationship. However, as we only found a positive relationship between LSM and cuticle PROTO IX concentration in "tinted" and "dark brown" eggs, with no association in "brown", "white" or "blue" eggs, we believe that a combination of both genetic and hormonally-regulated extrinsic factors are responsible for the observed relationship.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

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REFERENCES

Ahmed, B., C. De Boeck, A. Dumont, E. Cox, K. De Reu, and D. Vanrompay. 2017. First experimental evidence for the transmission of *Chlamydia psittaci* in poultry through eggshell penetration. Transbound. Emerg. Dis. 64:167–170.

Bai, D. P., X. Y. Lin, Y. Wu, S. Y. Zhou, Z. B. Huang, Y. F. Huang, A. Li, and X. H. Huang. 2019. Isolation of blue-green eggshell pigmentation-related genes from Putian duck through RNA-seq. BMC Genomics 20:66. doi 10.1186/s12864-019-5436-4

Bain, M. M., K. McDade, R. Burchmore, A. Law, P. W. Wilson, M. Schmutz, R. Preisinger, and I. C. Dunn. 2013. Enhancing the egg's natural defence against bacterial penetration by increasing cuticle deposition. Anim. Genet. 44:661–668.

Bain, M. M., J. X. Zheng, M. Zigler, N. Whenham, F. Quinlan-Pluck, A. C. Jones, M. Roberts, W. Icken, V. E. Olori, and I. C. Dunn. 2019. Cuticle deposition improves the biosecurity of eggs through the laying cycle and can be measured on hatching eggs without compromising embryonic development. Poult. Sci. 98:1775–1784.

Baron, F., and S. Jan. 2011. Egg and egg product microbiology. Pages 330–350 in Improving the Safety and Quality of Eggs and Egg Products. Y. Nys, M. Bain, and F. V. Immerseel eds. Woodhead Publishing Ltd, Cambridge, UK.

Baron, F., S. Jan, F. Gonnet, M. Pasco, J. Jardin, B. Giudici, M. Gautier, C. Guerin-Dubiard, and F. Nau. 2014. Ovotransferrin plays a major role in the strong bactericidal effect of egg white against the *Bacillus cereus* group. J. Food Protect. 77:955–962.

Bates, D., M. Machler, B. M. Bolker, and S. C. Walker. 2015. Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Softw. 67:1–48.

Bedrani, L., E. Helloin, N. Guyot, and Y. Nys. 2013a. Systemic administration of lipopolysaccharide in laying hens stimulates antimicrobial properties of egg white against *Staphylococcus aureus*. Vet, Immunol. Immunop. 152:225–236.

Bedrani, L., E. Helloin, N. Guyot, S. Rehault-Godbert, and Y. Nys. 2013b. Passive maternal exposure to environmental microbes selectively modulates the innate defences of chicken egg white by increasing some of its antibacterial activities. BMC Microbiol. 13:128. doi 10.1186 /1471-2180-13-128

Bi, H. J., Z. Liu, C. J. Sun, G. Q. Li, G. Q. Wu, F. Y. Shi, A. Q. Liu, and N. Yang. 2018. Brown eggshell fading with layer ageing: dynamic change in the content of protoporphyrin IX. Poult. Sci. 97:1948–1953.

Bilkova, B., Z. Swiderska, L. Zita, D. Laloe, M. Charles, V. Benes, P. Stopka, and M. Vinkler. 2018. Domestic Fowl Breed Variation in Egg White Protein Expression: Application of Proteomics and Transcriptomics. J. Agr. Food Chem. 66:11854–11863.

Butler, M. W., and K. J. McGraw. 2013. Eggshell coloration reflects both yolk characteristics and dietary carotenoid history of female mallards. Funct. Ecol. 27:1176–1185.

Butler, M. W., and H. S. Waite. 2016. Eggshell biliverdin concentration does not sufficiently predict eggshell coloration. J. Avian Biol. 47:491–499.

Carvalho, T. S. M., L. S. Sousa, F. A. Nogueira, D. P. Vaz, M. M. Saldanha, M. V. Triginelli, M. Pinto, N. C. Baiao, and L. J. C. Lara. 2018. Digestible methionine plus cysteine in the diet of commercial layers and its influence on the performance, quality, and amino acid profile of eggs and economic evaluation. Poult. Sci. 97:2044–2052.

Cassey, P., G. H. Thomas, S. J. Portugal, G. Maurer, M. E. Hauber, T. Grim, P. G. Lovell, and I. Miksik. 2012. Why are birds' eggs colourful? Eggshell pigments co-vary with life-history and nesting ecology among British breeding non-passerine birds. Biol. J. Linn. Soc. 106:657–672.

Chen, X., X. Z. Li, Y. Y. Guo, W. B. Li, J. L. Song, G. Y. Xu, N. Yang, and J. X. Zheng. 2019. Impact of cuticle quality and eggshell thickness on egg antibacterial efficiency. Poult. Sci. 98:940–948.

Chousalkar, K., and V. C. Gole. 2016. Salmonellosis acquired from poultry. Curr. Opin. Infect. Dis. 29:514–519.

Chousalkar, K., R. Gast, F. Martelli, and V. Pande. 2018. Review of egg-related salmonellosis and reduction strategies in United States, Australia, United Kingdom and New Zealand. Crit. Rev. Microbiol. 44:290–303.

Cox, J. M., and A. Pavic. 2010. Advances in enteropathogen control in poultry production. J. Appl. Microbiol. 108:745–755.

Crawley, M. J. 2007. The R Book. John Wiley & Sons, Ltd., New Jersey, USA.

D'Alba, L., R. Torres, G. I. N. Waterhouse, C. Eliason, M. E. Hauber, and M. D. Shawkey. 2017. What does the eggshell cuticle do? A functional comparison of avian eggshell cuticles. Physiol. Biochem. Zool. 90:588–599.

De Reu, K., W. Messens, M. Heyndrickx, T. B. Rodenburg, M. Uyttendaele, and L. Herman. 2008. Bacterial contamination of table eggs and the influence of housing systems. World. Poult. Sci. J. 64:5–19.

Dearborn, D. C., S. M. Page, M. Dainson, M. E. Hauber, and D. Hanley. 2017. Eggshells as hosts of bacterial communities: An experimental test of the antimicrobial egg coloration hypothesis. Ecol. Evol. 7:9711–9719.

Duval, C., P. Cassey, I. Miksik, S. J. Reynolds, and K. A. Spencer. 2013. Condition-dependent strategies of eggshell pigmentation: an experimental study of Japanese quail (*Coturnix coturnix japonica*). J. Exp. Biol. 216:700–708.

Gautron, J., S. Rehault-Godbert, G. Pascal, Y. Nys, and M. T. Hincke. 2011. Ovocalyxin-36 and other LBP/BPI/PLUNC-like

proteins as molecular actors of the mechanisms of the avian egg natural defences. Biochem. Soc. T. 39:971–976.
Giansanti, F., L. Leboffe, G. Pitari, R. Ippoliti, and G. Antonini. 2012. Physiological roles of ovotransferrin. BBA-Gen. Subjects 1820:218–225.
Giansanti, F., M. T. Massucci, M. F. Giardi, F. Nozza, E. Pulsinelli, C. Nicolini, D. Botti, and G. Antonini. 2005. Antiviral activity of ovotransferrin derived peptides. Biochem. Bioph. Res. Co. 331:69–73.
Hargitai, R., M. Herenyi, G. Nagy, and J. Torok. 2018. Eggshell spotting pattern is related to hatching asynchrony, hematocrit value and growth of nestling great tits *Parus major*. J. Avian Biol. 49: 10.1111/jav.01827
Hargitai, R., G. Nagy, M. Herenyi, Z. Nyiri, M. Laczi, G. Hegyi, Z. Eke, and J. Torok. 2016. Darker eggshell spotting indicates lower yolk antioxidant level and poorer female quality in the Eurasian Great Tit (*Parus major*). Auk 133:131–146.
He, T., H. J. Zhang, J. Wang, S. G. Wu, H. Y. Yue, and G. H. Qi. 2017. Proteomic comparison by iTRAQ combined with mass spectrometry of egg white proteins in laying hens (*Gallus gallus*) fed with soybean meal and cottonseed meal. PLoS One 12: e0182886, doi 10.1371/journal.pone.0182886
Horrocks, N. P. C., K. Hine, A. Hegemann, H. K. Ndithia, M. Shobrak, S. Ostrowski, J. B. Williams, K. D. Matson, and B. I. Tieleman. 2014. Are antimicrobial defences in bird eggs related to climatic conditions associated with risk of trans-shell microbial infection? Front. Zool. 11: doi 10.1186/1742-9994-11-49
Horrocks, N. P. C., B. I. Tieleman, and K. D. Matson. 2011. A simple assay for measurement of ovotransferrin - a marker of inflammation and infection in birds. Methods Ecol. Evol. 2:518–526.
Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous inference in general parametric models. Biom. J. 50:346–363.
Ibrahim, H. R., T. Matsuzaki, and T. Aoki. 2001. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett. 506:27–32.
Ibrahim, H. R., Y. Sugimoto, and T. Aoki. 2000. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. BBA-Gen. Subjects 1523:196–205.
Ibrahim, H. R., M. Yamada, K. Matsushita, R. Kobayashi, and A. Kato. 1994. Enhanced bactericidal action of lysozyme to Escherichia-coli by inserting a hydrophobic pentapeptide into its C-terminus. J. Biol. Chem. 269:5059–5063.
Ishikawa, S., K. Suzuki, E. Fukuda, K. Arihara, Y. Yamamoto, T. Mukai, and M. Itoh. 2010. Photodynamic antimicrobial activity of avian eggshell pigments. FEBS Lett. 584:770–774.
Joseph, N. S., N. A. Robinson, R. A. Renema, and F. E. Robinson. 1999. Shell quality and color variation in broiler breeder eggs. J. Appl. Poult. Res. 8:70–74.
Kennedy, G. Y., and H. G. Vevers. 1976. Survey of avian eggshell pigments. Comp. Biochem. Phys. B. 55:117–123.
Ketta, M., and E. Tumova. 2018. Eggshell characteristics and cuticle deposition in three laying hen genotypes housed in enriched cages and on litter. Czech J. Anim. Sci. 63:11–16.
Kinoshita, K., S. L. Myint, T. Shimogiri, H. R. Ibrahim, K. Kawabe, S. Okamoto, Y. P. Lee, Y. Matsuda, and Y. Maeda. 2016. Chicken ovotransferrin variants OTFB and OTFC harboring substitution of GAT (Asp) to AAT (Asn) in the codon 500 and their antimicrobial activity. J. Poult. Sci. 53:257–263.
Kozuszek, R., H. Kontecka, S. Nowaczewski, G. Lesnierowski, J. Kijowski, and A. Rosinski. 2009. Quality of pheasant (*Phasianus colchicus* L.) eggs with different shell colour. Arch. Geflugelkd. 73:201–207.
Kulshreshtha, G., A. Rodriguez-Navarro, E. Sanchez-Rodriguez, T. Diep, and M. T. Hincke. 2018. Cuticle and pore plug properties in the table egg. Poult. Sci. 97:1382–1390.
Li, G. Q., C. J. Sun, G. Q. Wu, F. Y. Shi, A. Q. Liu, and N. Yang. 2016. iTRAQ-based quantitative proteomics identifies potential regulatory proteins involved in chicken eggshell brownness. PLoS One 11: e0168750, doi 10.1371/journal.pone.0168750
Masschalck, B., and C. W. Michiels. 2003. Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. Crit. Rev. Microbiol. 29:191–214.
Maurer, G., S. J. Portugal, and P. Cassey. 2011. Review: an embryo's eye view of avian eggshell pigmentation. J, Avian Biol. 42:494–504.
Mertens, K., I. Vaesen, J. Loffel, B. Kemps, B. Kamers, C. Perianu, J. Zoons, P. Darius, E. Decuypere, J. De Baerdemaeker, and B. De Ketelaere. 2010. The transmission color value: a novel egg quality measure for recording shell color used for monitoring the stress and health status of a brown layer flock. Poult. Sci. 89:609–617.
Miksik, I., P. Ergang, and J. Pacha. 2014. Proteomic analysis of chicken eggshell cuticle membrane layer. Anal. Bioanal. Chem. 406:7633–7640.
Miksik, I., V. Holan, and Z. Deyl. 1996. Avian eggshell pigments and their variability. Comp. Biochem. Physiol. B. 113:607–612.
Myint, S. L., K. Kinoshita, T. Shimogiri, H. R. Ibrahim, T. Tsusaki, T. Tanoue, K. Kawabe, Y. Maeda, and S. Okamoto. 2012a. Effect of polymorphism in egg white lysozyme on muramidase and antibacterial activities as well as hatchability in the Japanese quail (*Coturnix japonica*). J. Anim. Sci. 90:1747–1755.
Myint, S. L., T. Shimogiri, K. Kinoshita, K. Nirasawa, N. Saitoh, H. Watanabe, K. Kawabe, Y. Maeda, and S. Okamoto. 2012b. Analysis of egg white lysozyme polymorphisms among Japanese Quail populations in Japan and France. J. Poult. Sci. 49:74–78.
Narushin, V. G. 2005. Production, modeling, and education - Egg geometry calculation using the measurements of length and breadth. Poult. Sci. 84:482–484.
Nowaczewski, S., T. Szablewski, R. Cegielska-Radziejewska, K. Stuper-Szablewska, M. Rudzinska, G. Lesnierowski, H. Kontecka, and K. Szulc. 2013. Effect of housing system and eggshell colour on biochemical and microbiological characteristics of pheasant eggs. Arch. Geflugelkd. 77:226–233.
Nys, Y., J. Gautron, J. M. Garcia-Ruiz, and M. T. Hincke. 2004. Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. C. R. Palevol 3:549–562.
Odabasi, A. Z., R. D. Miles, M. O. Balaban, and K. M. Portier. 2007. Changes in brown eggshell color as the hen ages. Poult. Sci. 86:356–363.
Osserman, E. F., and D. P. Lawlor. 1966. Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J. Exp. Med. 124:921–931.
Palmiter, R. D., E. R. Mulvihill, J. H. Shepherd, and G. S. McKnight. 1981. Steroid-hormone regulation of ovalbumin and conalbumin gene-transcription - a model based upon multiple regulatory sites and intermediary proteins. J. Biol. Chem. 256:7910–7916.
RStudio Team. 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA. http ://www.rstudio.com/.
Rubin, C. J., M. C. Zody, J. Eriksson, J. R. S. Meadows, E. Sherwood, M. T. Webster, L. Jiang, M. Ingman, T. Sharpe, S. Ka, F. Hallbook, F. Besnier, O. Carlborg, B. Bed'hom, M. Tixier-Boichard, P. Jensen, P. Siegel, K. Lindblad-Toh, and L. Andersson. 2010. Whole-genome resequencing reveals loci under selection during chicken domestication. Nature 464:587–U145.
Samiullah, S., A. S. Omar, J. Roberts, and K. Chousalkar. 2017. Effect of production system and flock age on eggshell and egg internal quality measurements. Poult. Sci. 96:246–258.
Samiullah, S., and J. R. Roberts. 2014. The eggshell cuticle of the laying hen. World Poult. Sci. J, 70:693–707.
Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9:671–675.
Sellier, N., M. L. Vidal, F. Baron, J. Michel, J. Gautron, M. Protais, C. Beaumont, M. Gautier, and Y. Nys. 2007. Estimations of repeatability and heritability of egg albumen antimicrobial activity and of lysozyme and ovotransferrin concentrations. Brit. Poult. Sci. 48:559–566.
Soh, T., N. Fujihara, and O. Koga. 1993. Observations of pigment accumulation in the epithelium of the shell gland and superficial pigmentation on the egg-shell in japanese-quail. J. Fac. Agr. Kyushu U. 38:73–80.
Soh, T., and O. Koga. 1994. The effects of sex steroid-hormones on the pigment accumulation in the shell gland of japanese-quail. Poult. Sci. 73:179–185.

Soler, J. J., C. Ruiz-Castellano, J. Figuerola, J. Martinez-de la Puente, M. Ruiz-Rodriguez, and G. Tomas. 2018. Egg colouration predicts brood size, telomere length and body condition of spotless starling fledglings. J. Avian Biol. 49: doi 10.1111/jav.01686

Stevens, L. 1991. Egg-white proteins. Comp. Biochem. Physiol. B. 100:1–9.

Sun, C. J., J. N. Liu, W. B. Li, G. Y. Xu, and N. Yang. 2017. Divergent proteome patterns of egg albumen from domestic chicken, duck, goose, turkey, quail and pigeon. Proteomics 17:12. doi 10.1002/pmic.201700145

Svobodová, J., L. Šmídová, and V. Gvoždíková Javůrková. 2019. Different incubation patterns affect selective antimicrobial properties of the egg interior: experimental evidence from eggs of precocial and altricial birds. J. Exp. Biol. 222: jeb201442. doi:10.1242/jeb.201442

Ugurlu, M., Y. K. Das, F. Akdag, E. Atmaca, M. Salman, B. Teke, and S. Arslan. 2017. Effect of egg weight and amount of protoporphyrin and biliverdin in the egg shell on hatching characteristics and embryonal mortality in pheasants (*Phasianus colchicus*). Ankara Univ. Vet. Fak. 64:117–124.

Vlckova, J., E. Tumova, M. Ketta, M. Englmaierova, and D. Chodova. 2018. Effect of housing system and age of laying hens on eggshell quality, microbial contamination, and penetration of microorganisms into eggs. Czech J. Anim. Sci. 63:51–60.

Vlckova, J., E. Tumova, K. Mikova, M. Englmaierova, M. Okrouhla, and D. Chodova. 2019. Changes in the quality of eggs during storage depending on the housing system and the age of hens. Poult. Sci. pez401. doi.org/10.3382/ps/pez401

Wang, Z. P., G. H. Meng, Y. Bai, R. F. Liu, Y. Du, and L. H. Su. 2017. Comparative transcriptome analysis provides clues to molecular mechanisms underlying blue-green eggshell color in the Jinding duck (*Anas platyrhynchos*). BMC Genomics 18. doi 10.1186/s12864-017-4135-2

Wang, Z. P., L. J. Qu, J. F. Yao, X. L. Yang, G. Q. Li, Y. Y. Zhang, J. Y. Li, X. T. Wang, J. R. Bai, G. Y. Xu, X. M. Deng, N. Yang, and C. X. Wu. 2013. An EAV-HP insertion in 5 ' flanking region of SLCO1B3 causes blue eggshell in the chicken. PLoS Genet. 9. doi 10.1371/journal.pgen.1003183

Wellman-Labadie, O., J. Picman, and M. T. Hincke. 2008a. Antimicrobial activity of cuticle and outer eggshell protein extracts from three species of domestic birds. Brit. Poultry Sci. 49:133–143.

Wellman-Labadie, O., J. Picman, and M. T. Hincke. 2008b. Comparative antibacterial activity of avian egg white protein extracts. Brit. Poultry Sci. 49:125–132.

Wellman-Labadie, O., J. Picman, and M. T. Hincke. 2008c. Enhanced c-type lysozyme content of wood duck (*Aix sponsa*) egg white: An adaptation to cavity nesting? Physiol. Biochem. Zool. 81:235–245.

Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York, USA.

Yamanishi, H., S. Iyama, Y. Yamaguchi, Y. Kanakura, and Y. Iwatani. 2002. Modification of fully automated total iron-binding capacity (TIBC) assay in serum and comparison with dimension TIBC method. Clin. Chem. 48:1565–1570.

Yang, H. M., Z. Y. Wang, and J. Lu. 2009. Study on the relationship between eggshell colors and egg quality as well as shell ultrastructure in Yangzhou chicken. Afr. J. Biotechnol. 8:2898–2902.

Zhang, L., D. M. Chen, L. T. Yu, Y. Wei, J. Li, and C. Q. Zhou. 2019. Genome-wide analysis of the ovodefensin gene family: Monophyletic origin, independent gene duplication and presence of different selection patterns. Infect. Genet. Evol. 68:265–272.

Zhang, M. M., L. Yang, Z. C. Su, M. Z. Zhu, W. T. Li, K. L. Wu, and X. M. Deng. 2017. Genome-wide scan and analysis of positive selective signatures in Dwarf Brown-egg Layers and Silky Fowl chickens. Poult. Sci. 96:4158–4171.

Zheng, C. W., Z. S. Li, N. Yang, and Z. H. Ning. 2014. Quantitative expression of candidate genes affecting eggshell color. Anim. Sci. J. 85:506–510.