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# New insights into the relationships between egg maternal components: the interplays between albumen steroid hormones, proteins and eggshell protoporphyrin

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ABSTRACT

Recent studies have shown that the egg yolk maternal components, which are a mixture of substances that can affect the developing embryo, do not act separately but are interconnected and co-adapted. Surprisingly, no study to date has focused on the associations between maternally derived albumen steroids and albumen and eggshell compounds with pleiotropic effects. Eggshell pigment protoporphyrin (PROTO IX) should provide primary antimicrobial protection for eggs, but as a proven pro-oxidant, it may compromise female fitness. Abundant albumen proteins ovotransferrin (OVOTR) and lysozyme (LSM) have been shown to have antimicrobial, antioxidant, immunoregulatory and growth-regulatory roles. To investigate associations between albumen steroids and OVOTR, LSM and eggshell cuticle PROTO IX, we used chicken eggs with differently pigmented eggshells. We found that albumen steroid hormones were strongly intercorrelated. In addition, we revealed that albumen LSM and testosterone (T) were positively associated, while a negative association was found between albumen LSM and pregnenolone (P5). Eggshell cuticle PROTO IX was negatively associated with the concentration of albumen 17α-hydroxypregnenolone (17-OHP5). Finally, of all the hormones tested, only the concentration of albumen 17-OHP5 correlated negatively with egg volume and varied with eggshell colour and chicken breed. Although experimental evidence for the effect of maternal albumen steroids on avian developing embryo is still scarce, our study is the first to highlight co-variation and potential co-adjustment of maternally derived albumen steroids, proteins and eggshell cuticle pigment suggesting similar allocation mechanisms known for yolk maternal compounds with the potential to influence the avian embryo and offspring phenotype.

## 1. Introduction

In birds, females allocate maternal resources into their eggs, which are distributed into the various egg structures (i.e. yolk and albumen) where they form the basis for an orchestra of biochemical processes within the egg (Huopalahti et al., 2007). However, the "allocation strategy" may differ for different egg components. It is suggested that the deposition of nutritionally and physiologically costly compounds (e.g. lipids and antibodies) into the egg is more controlled by the nutritional (e.g. diet or food availability) and/or physiological constraints of the female (e.g. age, condition, immunity), who may compensate for the allocation of these compounds by depositing energetically or physiologically less demanding compounds such as hormones and proteins, which may in turn be more beneficial to the embryo or offspring (Groothuis et al., 2006; Valcu et al., 2019; Mentesana et al., 2021). This leads to the assumption that allocation and distribution of individual egg components is the result of passive mechanisms linked to changes in physiological and biochemical processes within the female body during egg formation (Williams and Groothuis, 2015; Valcu et al., 2019). Alternatively, active investment more controlled by female regulatory and transfer mechanisms resulting from adaptation to specific conditions and responsible for targeted allocation of specific compounds have also been observed (Badyaev et al., 2006; Groothuis et al., 2006). Although it is still unclear to what extent non-mutually exclusive passive and active mechanisms are involved in the allocation of specific maternally derived substances to the egg, most of these substances affect

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the embryo and act as so-called maternal effects (Groothuis and Schwabl, 2008).

This is particularly true for the yolk compounds ranging from steroid and thyroid hormones to antibodies and fatty acids which have been shown to affect avian offspring during both the embryonic (Groothuis et al., 2005; von Engelhardt and Groothuis, 2011; Williams and Groothuis, 2015; Ruuskanen et al., 2016, 2017; Sarraude et al., 2020; Mentesana et al., 2021; Hsu et al., 2017, 2022) and also post-hatching periods (Hegyi et al., 2011; Hsu et al., 2016). In addition, co-variation and compensatory fine-tuning of synergistically or antagonistically acting maternally deposited yolk components able to eliminate the deleterious pro-oxidant effects of androgens through increased deposition of antioxidants (Navara et al., 2006; Giraudeau and Ducatez, 2016), carotenoids (Giraudeau et al., 2017) or antibodies (Partecke et al., 2020) has been documented. It seems therefore that the mother is most likely able to co-adapt egg yolk maternal components, probably as a result of both passive and active compensatory allocation mechanisms (Groothuis et al., 2006; Giraudeau and Ducatez, 2016; Parolini et al., 2017; Possenti et al., 2018; Valcu et al., 2019; Mentesana et al., 2021).

The preference for studying predominantly yolk lipophilic substances with high nutritional value and permeability index to embryonic membranes is understandable. However, relationships between the substances contained in the albumen and cuticle of the eggshell, which are rather lipophilic proteins with a lower membrane permeability but forming an important defence system of the developing embryo, should not be neglected. As the eggs in the clutch are in constant and close contact with ubiquitous microorganisms occurring in high abundance and diversity on the eggshell (Peralta-Sanchez et al., 2012; Grizard et al., 2014, 2015), penetrating inside the egg (Javurkova et al., 2014), fundamentally influencing egg hatchability (Cook et al., 2003, 2005; Peralta-Sanchez et al., 2018) and the resulting chick phenotype (Javurkova et al., 2014), birds protect their eggs from these negative effects of microorganisms by various mechanisms. These may include deposition of antimicrobial proteins (AMPs) or pigment protoporphyrin IX (PROTO IX) into the eggshell cuticle hypothesized to protect proliferation of microorganisms on the egg surface (Ishikawa et al., 2010; Wellman-Labadie et al., 2008a; D'Alba et al., 2017), and plethora of antimicrobial proteins (AMPs) contained in the albumen protecting the embryo from the effects of microorganisms penetrating from the eggshell into the egg interior (Wellman-Labadie et al., 2008b; Javurkova et al., 2019). The antimicrobial effect of the most abundant albumen AMPs, ovotransferrin (OVOTR), which has a very broad antimicrobial and antiviral spectrum of action (Giansanti et al., 2005; Wu and Acero-Lopez, 2012; Baron et al., 2014), and lysozyme (LSM), which is predominantly bactericidal against G+ bacteria (Sellier et al., 2007; Wellman-Labadie et al., 2008b), may differ between precocial and altricial species and along with the different incubation patterns of these species (Svobodova et al., 2019). However, OVOTR may be beneficial to the developing embryo also as an antioxidant (Ibrahim et al., 2007), immunomodulator (Xie et al., 2002) or as an important iron transporter due to its ability to bind to red blood cells (Ibrahim et al., 2000). Similarly, LSM has been shown to have a growth regulatory role (Kuettner et al., 1970; Sakamoto et al., 1974), where elevated concentrations of albumen LSM have led to the production of chicks with reduced tarsus length in quails (Javurkova et al., 2015) or reduced scale body mass in mallards (Svobodova et al., 2021). Thus, allocation of albumen AMPs may benefit the developing embryo by providing an antimicrobial, antioxidant or immunomodulatory function, while some may reduce embryo growth. However, whether these pleiotropic, and non-mutually exclusive roles of albumen AMPs are in a synergistic or compensatory relationship with other maternally derived albumen compounds has not yet been investigated. Furthermore, synthesis of albumen AMPs in oviduct and PROTO IX in the shell gland has been found to be hormonally regulated (Le Bouc et al., 1985; Soh and Koga, 1997; Kim and Choi, 2014) and growing number of studies have documented the presence of androgens, glucocorticoids and other various steroid hormones in albumen in concentrations almost equivalent to those found in the yolk (Downing and Bryden, 2008; Janczak et al., 2009; Poisbleau et al., 2009; De Baere et al., 2015; Caulfield and Padula, 2020). Questions therefore arise as to whether and to what extent albumen steroid hormones, AMPs and eggshell cuticle PROTO IX are intercorrelated. In addition, PROTO IX content in eggshell structures was found to be related to the genotype (Lu et al., 2021; Yang et al., 2022) and the concentration of AMPs in the albumen (Javurkova et al., 2019). Similarly, egg volume was found as significant predictor of different allocation of yolk compounds (Royle et al., 2001; Gil, 2003; Rubolini et al., 2011; Bebbington and Groothuis, 2021). Therefore, when examining the relationships between maternally-derived compounds in the albumen, these female- and egg-related factors must also be considered.

In this study, we used chicken eggs with differently pigmented eggshells to investigate the relationships between five maternally derived albumen steroid hormones (testosterone - T, androstenedione - A4, progesterone - P4, pregnenolone - P5 and 17α-hydroxypregnenolone - 17OHP5), two most abundant albumen proteins (LSM and OVOTR) with antimicrobial, antioxidant, immunomodulatory and growth-regulatory roles and PROTO IX, an antimicrobial, yet pro-oxidant substance deposited into the eggshell cuticle. Using a correlational approach, we sought to uncover whether, and to what extent these albumen maternal compounds are intercorrelated with the following assumptions: i) considering the hormonal regulation of albumen AMPs and eggshell cuticle PROTO IX synthesis, we expect that the concentrations of albumen AMPs and cuticle PROTO IX should be correlated with albumen steroids, which could reflect the hormonal status of the female at the time of egg formation; ii) given the low nutritional and physiological demands of steroid hormones synthesis, we expect rather weak or no effect of egg volume, advertising the possibility of resource allocation to the egg by the female, on the concentration of albumen steroid hormones; iii) since in a previous study we showed a combined genotype- and environmental and diet condition-dependent allocation of PROTO IX to eggshell cuticle and AMPs to albumen (Javurkova et al., 2019), we expect that chicken genotype and PROTO IX cuticle content will also be related to the concentration of steroids in albumen.

## 2. Material and methods

### 2.1. Ethical statement

The experimental procedure was approved by the Ethical Committee of the Faculty of Science of the Charles University (Permit No. 13060/2014-MZE-17214).

### 2.2. Egg collection and processing

In total, we processed 50 freshly laid eggs belonging to 19 traditional chicken breeds that were obtained in spring 2015 from non-commercial breeders in the Czech Republic (see Supplementary Table S1 for details). To account for the effect of temperature and incubation on the modulation of egg yolk and albumen biochemistry (e.g. Ruuskanen et al., 2016; Svobodova et al., 2019), all eggs were un-incubated and stored at a constant temperature of 23 °C for a maximum of 4 days and then processed as follows.

The length and width of each egg was measured to the nearest 0.01 mm using a Quatros® QS15506 digital scale (Lublin, Poland) to calculate egg volume (Narushin, 2005). Eggs were then assigned to one of the eggshell colour categories (i.e. blue, white, tinted, brown and dark brown) based on the intensity of blue/brown pigmentation (Supplementary Table S1). The different eggshell colour categories, represented by the respective chicken breeds, differed significantly in the mean concentration of PROTO IX in the eggshell cuticle, as presented in our previous study (Supplementary Fig. S1 and (Javurkova et al., 2019).

The eggshell cuticle was then extracted from each egg according to

the method of (Kennedy and Vevers, 1976), and which is described in detail in our previous study (Javurkova et al., 2019). Briefly, 40 mL of a 5% EDTA solution (Sigma-Aldrich GmbH, Steinheim, Germany) and 10 mM of 2-mercaptoethanol solution (PENTA, Prague, Czech Republic) buffered with NaOH to pH = 7.4 were used to immerse the egg in this solution for 1 h. The cuticle was then manually scraped with a plastic scalpel and the released cuticle in ddH2O was then dialyzed with the Pur-A-Lyzer™ Mega Dialysis Kit (Sigma-Aldrich GmbH, Steinheim, Germany) and lyophilized with Alpha 1–2 LD plus (Martin Christ GmbH, Osterode am Harz, Germany). The weighed lyophilized cuticle was then stored at −80 °C until PROTO IX analysis. Then, each egg was washed with ddH2O to remove any residual EDTA/Mercaptoethanol solution, dried, broken manually and the yolk and white carefully separated. The albumen was transferred to a 50 mL cryotube (NuncTM, Thermo-Fisher Scientific, Waltham, MA, USA) and stored at −80 °C until LSM and OVOTR analysis.

### 2.3. Analysis of albumen antimicrobials

#### 2.3.1. Albumen lysozyme

The concentration of egg white LSM was measured using a slightly modified agar well- diffusion method of (Osserman and Lawlor, 1966) already applied in several of our previous studies (Javurkova et al., 2015, 2019; Svobodova et al., 2019). Briefly, 50 mg of lyophilized *Micrococcus lysodeikticus* (ATTC 4698, M3770, Sigma-Aldrich GmbH, Steinheim, Germany) resuspended in 10 mL of Britton-Robinson buffer was added to a 60 °C solution of 1% agar (Alchimica, Prague, Czech Republic) prepared from Britton-Robinson buffer (see Javurkova et al., 2019). The agar was poured into Petri dishes, allowed to solidify for 30 min, and then 3-mm diameter holes were punched in the agar. The wells were then loaded with 10 μL of albumen samples homogenized with a magnetic stirrer for 30 min at 1800 rpm. All samples were loaded in duplicates. Standard solutions of LSM (10 μL) of known concentrations (20, 15, 7, 4, 2, 0.5 mg/mL) prepared by diluting lyophilized LSM from hen egg white (HEWL, L6876, Sigma-Aldrich, St. Louis, MO, USA) in Britton-Robinson buffer were placed in the wells of each petri dish. The plates were then incubated for 24 h at 21 °C and 50–60% humidity. Inhibition zone diameters were analysed using ImageJ 1.42q software (Schneider et al., 2012) from standardized photographs of plates (see Javurkova et al., 2019 for details). LSM concentration (mg/mL) analyses were performed by interpolating measured inhibition zones against calibration curves using GraphPad Prism v. 6.00 for Windows (GraphPad Software, San Diego California USA).

#### 2.3.2. Albumen ovotransferrin

OVOTR concentration was measured using a modified method of (Yamanishi et al., 2002) and described in detail in (Horrocks et al., 2011) and in our previous studies (Javurkova et al., 2019; Svobodova et al., 2019). Briefly, 25 μL of homogenized albumen sample (see above) was transferred into a 96-well microplate (BRAND® microplate, pureGrade, flat-transparent, Sigma-Aldrich, St. Louis, MO, USA) in quadruplicate. Similarly, 25 μL of OVOTR standard solutions with concentrations ranging from 30 mg/mL to 0.1 mg/mL prepared from OVOTR stock solution containing 40 mg of OVOTR (Conalbumin, C0755, Sigma-Aldrich, St. Louis, MO. Louis, MO, USA) dissolved in "reagent 1" containing 200 mL of ddH2O, 7.3 g of Tris, 6.4 g of Na2CO3, 0.84 g of Triton-X (Sigma-Aldrich, St. Louis, MO, USA) was transferred to the wells of the bottom rows of the plate in duplicates. Then, 120 μL of "reagent 2" containing 150 mL of "reagent 1" and 600 μL of iron standard solution (VWR International, Lutterworth, England) were added to each well, after 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, 25 μL of ascorbic acid solution (100 mL ddH2O, 0.49 g FerroZine™, 0.6 g Tris, 0.574 g ascorbic acid) was added to each well and placed 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 absorbance (570 nm and 660 nm wavelength) was recorded immediately ($t = 0$) and after a further 6 min of incubation at 37 °C ($t = 7$). The absorbance values were corrected to the initial well-specific absorbance 'pre-read' values and normalized using reference absorbance values at 660 nm wavelength. The difference between the values measured at $t = 0$ and t = 7 was used to calculate the OVOTR concentration by interpolating the standard curve in GraphPad Prism v. 6.00 for Windows (GraphPad Software, San Diego California USA).

### 2.4. Analysis of albumen steroids

Analysis of the concentration of five albumen steroid hormones (androstenedione – A4, progesterone – P4, testosterone – T, pregnenolone – P5 and 17α-hydroxypregnenolone – 17-OHP5 from 50 egg white samples was performed in service laboratory (Department of Steroids and Proteofactors, Institute of Endocrinology in Prague, Czech Republic) using a liquid chromatography with mass detection (LC - MS/MS) according to the protocol of Sosvorova et al. (2015).

First, all necessary deuterated standard and internal standard (ISTD) solutions were pipetted into all empty glass extraction tubes and evaporated in a vacuum evaporator to dryness. Standard curve samples, zero samples (only internal standards added) and blank samples (no standards added) were prepared in duplicate similarly for the albumen samples.

Then, 2 mL of albumen sample homogenized by shaking at 1200 rpm 10 min was transferred to clean plastic tube with 3 mL of LC-MS grade water, mixed at 1200 rpm 10 min and 1250 μL of such prepared sample was extracted with 2 mL of diethyl ether. Organic phase was transferred into the glass extraction tube and the solvent was evaporated.

In the next step, the sample was incubated with the derivatization solution (see Sosvorova et al., 2015 for details) for 15 min at 60 °C and evaporated with a stream of nitrogen. The dried residue was dissolved in 100 μL of 10 mM ammonium formate solution in 50% methanol and mixed vigorously. The sample was then centrifuged (2000 rpm, 4 min, 22 °C), and the whole amount of solvent was transferred to the insert vial and injected into the UPLC. Subsequently, LC - MS/MS was performed using an API 3200 (AB Sciex, Concord, Canada) triple stage quadrupole-mass spectrometer with electrospray ionization (ESI) connected to the UPLC Eksigent ultraLC 110 system (Redwood City, CA, USA). Chromatographic separation was carried out on Kinetex C18 2.6 lm (150 × 3.0 mm) column (Phenomenex, Torrance, CA, USA) with a corresponding security guard at the flow rate 0.55 mL/min at 40 °C. The analytes were quantified (ng/mL) by calibration curves based on known concentrations in the mixtures of the standards analysed with constant ISTD levels.

### 2.5. Concentration of eggshell cuticle protoporphyrin IX

PROTO IX was determined in the form of dimethyl ester, using the method of (Miksik et al., 1996) for determination of porphyrins in eggshell and described in detail in our previous study (Javurkova et al., 2019). Briefly, samples were extracted (and esterified) from the eggshell cuticles 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 for two days. The extracts were then filtrated (see Miksik et al., 1996 for details) 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 two 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 5u C18 110A column (250 × 2.0 mm I.D., Phenomenex, Torrence, CA, USA). For detailed parameter settings of the whole analytical procedure see Javurkova et al. (2019).

### 2.6. Statistical analysis

Since the effect of eggshell colour, chicken breed and egg volume on the concentration of AMPs in albumen and PROTO IX in the eggshell cuticle has been tested and documented in our previous study (Javurkova et al., 2019), we limited our analysis to testing the effect of these factors only on the concentration of five albumen steroids.

First, the effect of eggshell colour, chicken breed and egg volume on the concentration of five albumen steroid hormones was tested using three separate one-way Multiple Analyses of Variance (MANOVA). Steroid hormone concentrations were power transformed except for progesterone, where a reciprocal transformation [(i.e. original values were replaced by the inverse of x (−1/x)] was used to achieve data normality. Instead of a method using significance tests based on stepwise procedures, the results of full MANOVAs are presented as the statistically most robust (Mundry and Nunn, 2009; Korner-Nievergelt et al., 2015). As this analysis revealed a significant effect (all $P < 0.001$) of all three explanatory variables (i.e. eggshell colour, chicken breed and egg volume) but only on the concentration of albumen 17-OHP5 (Table 1), we first examined the effect size for each explanatory variable using one-way ANOVA. This analysis revealed the strongest effect size for egg volume (Eta (η)2 = 0.58), followed by breed (Eta (η)2 = 0.44) and eggshell colour with the lowest effect size (Eta (η)2 = 0.22). However, as additional ANOVA test revealed egg volume explained by the both eggshell colour (ANOVA: $df = 4$, $F = 68.359$, $P < <0.001$; Supplementary Fig. S1) and chicken breed (ANOVA: $df = 14$, $F = 6.164$, $P \leq <0.001$; Supplementary Fig. S1), to further examine the effect of egg volume on the concentration of albumen 17-OHP5 and to account for this data non-independence, we used the Linear Mixed Effects Model (LMM) implemented in the *lme4* R package (Bates et al., 2015) with eggshell colour nested in chicken breed as random effects in LMM.

Furthermore, we used bivariate Pearson correlation analysis to test and visualize the strength, direction and statistical significance (α = 0,05) of linear relationships between concentration of five albumen steroid hormones, AMPs and eggshell cuticle PROTO IX content using r packages *Hmisc* (Harrell and Dupont, 2019), *corrplot* (Wei and Simko, 2021) and *ggplot2* (Wickham, 2016). LSM, OVOTR and PROTO IX concentrations were log-transformed and steroid hormone concentrations were power transformed except for progesterone, where a reciprocal transformation [(i.e. original values were replaced by the inverse of x (−1/x)] was used to achieve normal distribution of data.

All analyses were performed using the R software (R Development Core Team, 2017) and Rstudio version 1.1.453 (RStudioTeam, 2015).

## 3. Results

### 3.1. The role of egg volume, eggshell colour, and chicken breed on the concentration of albumen steroid hormones

We found that only the concentration of albumen 17-OHP5 varied with eggshell colour, chicken breed, and egg volume (Table 1), with the strongest effect of egg volume (effect size of one-way ANOVA: Eta (η)2 = 0.58), followed by chicken breed (effect size of one-way ANOVA: Eta (η)2 = 0.44; Supplementary Fig. S2) and eggshell colour (effect size of one-way ANOVA: Eta (η)2 = 0.22; Supplementary Fig. S2). The relationship between egg volume and concentration of albumen 17-OHP5 remained highly significant even after controlling for the effects of eggshell colour and chicken breed (LMM: $df = 1$, $F = 10.96$, $P < 0.001$) and showed that smaller eggs had higher concentrations of albumen 17-OHP5 and vice versa (Pearson correlation: $t = -4.32$, $r = -0.53$, $P \leq <0.0001$; Fig. 1). For the other albumen steroids, the effects of egg volume, eggshell colour and chicken breed remained insignificant (Table 1).

### 3.2. Relationships between albumen steroid hormones, antimicrobials and eggshell cuticle protoporphyrin IX

Concentrations of albumen steroids varied from each other with pregnenolone - P5 showing the highest concentration (mean ± SE = 9.06 ± 4.85 ng/mL), followed by testosterone - T (mean ± SE = 0. 86 ± 0.71 ng/mL), androstenedione - A4 (mean ± SE = 0.84 ± 0.64 ng/mL), 17α-hydroxypregnenolone - 17-OHP5 (mean ± SE = 0.20 ± 0.20 ng/mL) and progesterone - P4 showing the lowest concentration (mean ± SE = 0.13 ± 0.06 ng/mL). Mean ± SE concentration of albumen LSM and OVOTR was 12.95 ± 3.66 (mg/mL) and 1.47 ± 1.34 (mg/mL), respectively.

Albumen steroids were strongly intercorrelated (Fig. 2A), with androstenedione – A4 showing a significant positive association with testosterone – T (Pearson correlation: $r = 0.65$, $P < 0.001$; Fig. 2A, B) and pregnenolone – P5 (Pearson correlation: $r = 0.35$, $P < 0.05$; Fig. 2A, B), while it showed a significant negative association with progesterone – P4 (Pearson correlation: $r = -0.42$, $P < 0.01$; Fig. 2A, B).

Whereas albumen LSM and OVOTR concentrations were not associated (Pearson correlation: $r = 0.01$; Fig. 2A), LSM was positively associated with testosterone – T (Pearson correlation: $r = 0.39$, $P < 0.05$; Fig. 2A, B), and negatively associated with pregnenolone – P5 (Pearson correlation: $r = -0.37$, $P < 0.05$; Fig. 2A, B) in albumen.

The PROTO IX content in the eggshell cuticle showed significant negative association with only albumen concertation of 17OHP5 (Pearson correlation: $r = -0.41$, $P < 0.05$; Fig. 2A, B), with no significant associations with other albumen compounds (Fig. 2B).

## 4. Discussion

In our study, we found that only 17-OHP5 albumen concentration was negatively correlated with egg volume (i.e. egg size) even after

**Table 1**
Results of three separate MANOVA models evaluating the effect of eggshell colour, egg volume and chicken breed on the concentration of five albumen steroid hormones in chicken eggs ($n = 48$). Significant predictors (α ≤ 0.05) in the full MANOVA models are shown in bold.

| Explanatory | EGGSHELL COLOUR | | | EGG VOLUME | | | CHICKEN BREED | | |
|---|---|---|---|---|---|---|---|---|---|
| Response | *df* | *F* | *P* | *df* | *F* | *P* | *df* | *F* | *P* |
| Progesterone | 4 | 0.777 | 0.546 | 1 | 0.101 | 0.753 | 18 | 1.170 | 0.342 |
| Pregnenolone | 4 | 0.789 | 0.539 | 1 | 2.503 | 0.120 | 18 | 0.799 | 0.686 |
| **17α-hydroxypregnenolone** | 4 | 11.118 | **<0.001*****| 1 | 19.634 | **<0.001***** | 18 | 4.535 | **<0.001***** |
| Testosterone | 4 | 0.692 | 0.601 | 1 | 3.219 | 0.051 | 18 | 1.815 | 0.072 |
| Androstenedione | 4 | 0.207 | 0.933 | 1 | 0.118 | 0.732 | 18 | 0.959 | 0.525 |

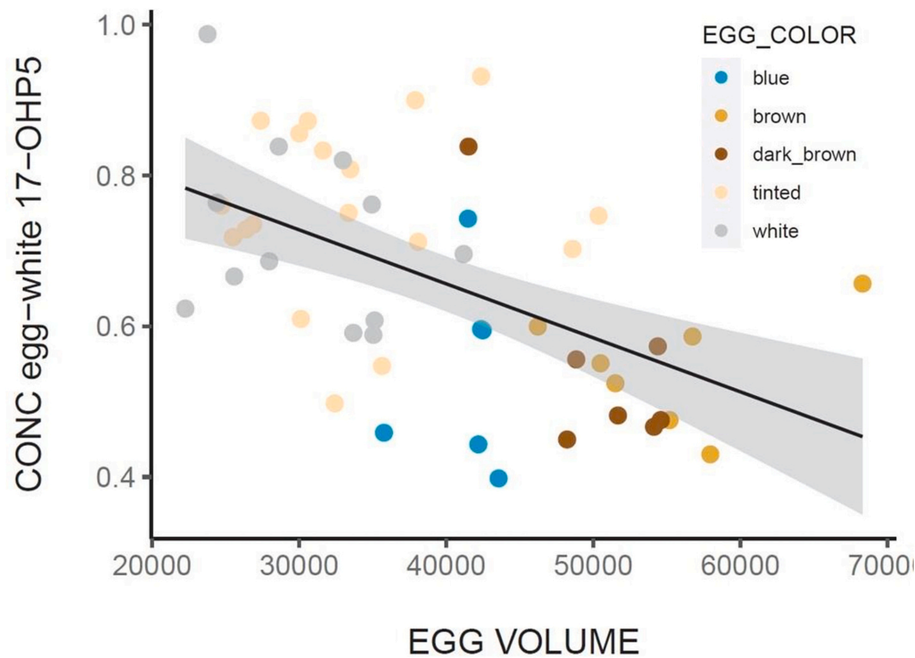


**Fig. 1.** Relationship between egg volume and the concentration of albumen 17α-hydroxypregnenolone (17-OHP5). Eggs with different eggshell colours are depicted by different colours in the scatterplot. Line and shaded area correspond to the linear regression slope and 95% CI. Values of 17-OHP5 on y-axis are power-transformed (17-OHP5 conc. (ng/ml) ˆ 0.2).

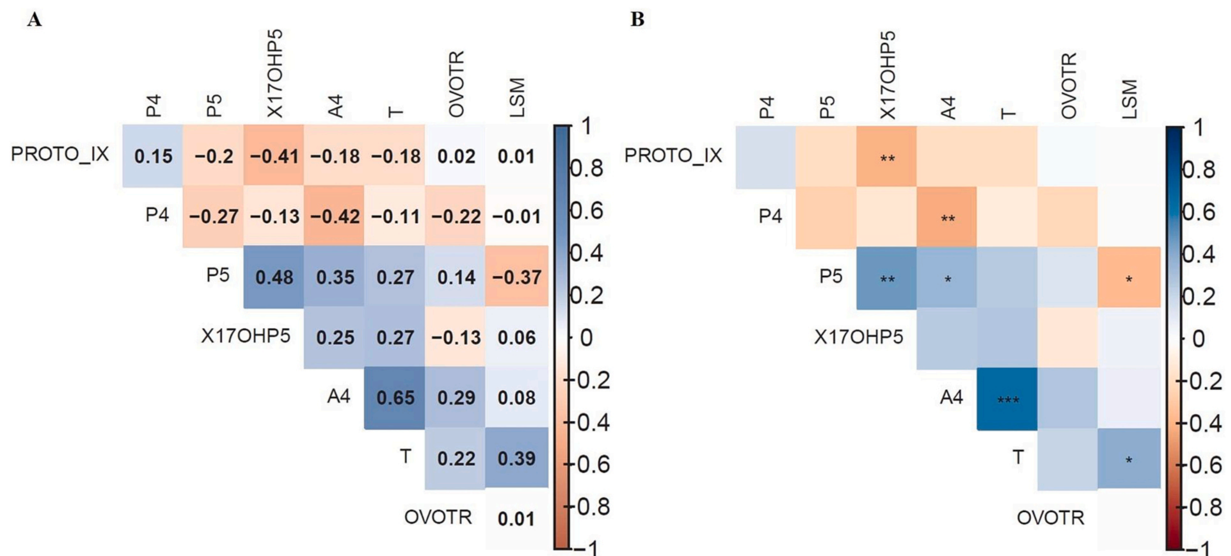


**Fig. 2.** Correlation matrices with A all Pearson correlation coefficients and B only significant correlations (* P < 0.05; ** P < 0.01; *** P < 0.001) showing associations and their significance between the concentrations of five albumen steroid hormones (T, A4, P4, P5, 17-OHP5), two albumen AMPs (LSM and OVOTR) and eggshell cuticle pigment protoporphyrin IX (PROTO IX). Positive correlations are shown in blue and negative correlations in red. Colour intensity are proportional to Pearson correlation coefficients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

controlling for the effects of eggshell pigmentation and chicken breed. This is consistent with our expectation that in the case of a passive allocation strategy, which assumes low energy requirements for the production of steroids whose circulating levels result only from standard unstimulated physiological processes of females at the time of egg formation, the allocation of steroids to albumen will be less affected by constraints on female investment in egg production. On the other hand, there are a number of studies showing that females use a compensatory allocation strategy and allocate various substances to smaller eggs that are known to produce lower quality offspring (Williams, 1994; Krist, 2011) to compensate for the reduced hatchling phenotype in other ways (Royle et al., 2001; Gil, 2003; Bebbington and Groothuis, 2021). Indeed, increased androgens allocation that accelerate growth and decreased allocation of carotenoids and vitamin E to the yolk of eggs with reduced egg mass decreasing along with laying order was observed in yellow-legged gulls (*Larus michahellis*) (Rubolini et al., 2011). Similarly, females of long-lived cockatiels (*Nymphicus hollandicus*) whose clutches hatch very asynchronously and egg mass decreased linearly with laying order, distributed yolk testosterone to reinforce the brood hierarchy due to asynchronous hatching (Kozlowski and Ricklefs, 2010). Even in megapode species with no parental care and sibling competition, the Australian Brush-turkey (*Alectura lathami*), higher deposition of

maternal T and A4 to the yolk in smaller eggs and higher deposition of T to eggs from larger mounds and laid at greater depths have been documented to compensate for the longer duration and higher energy requirements of the chicks during digging them out (Goth et al., 2008). In our study, we did not find support for such compensatory allocation mechanisms for albumen steroid hormones, as only the concentration of a previously understudied steroid in the context of maternal components deposited in the egg, 17-OHP5, increased with decreasing egg mass. Since the role of albumen steroids in modulating the developing embryo has not yet been sufficiently investigated, we can only assume possible maternal effects of the albumen 17-OHP5. For example, several studies have found that P5 itself and its derivatives formed by hydroxylation of P5, including 17-OHP5, are most recently considered to be very potent non-genomic neuromodulators in vertebrates (Matsunaga et al., 2001, 2002; Tsutsui et al., 2013a, 2013b; Weng and Chung, 2016; Mouton and Duckworth, 2021) with a very wide range of effects from modulation of migration (Wingfield et al., 2018), circadian activity, diurnal locomotor rhythms, and sexual behaviour through interaction with melatonin (Tsutsui et al., 2012; Ogura et al., 2016) to regulating locomotor activity in birds (Tsutsui et al., 2012, 2018). Given that increased locomotion ability just after hatching may be particularly crucial for precocial avian species (Belnap et al., 2019; Wu et al., 2022), it is possible to assume that increased deposition of the locomotion- and cognition-promoting 17-OHP5 into the albumen of lower-mass eggs might represent maternal compensation for reduced morphological traits in chicks originating from lower-mass eggs. However, as our study is purely correlative and the pathway for the effect of albumen 17-OHP5 on the avian embryo is not yet known, we can only hypothesize a potential effect of maternal albumen 17-OHP5 on the avian progeny until all these assumptions have been thoroughly investigated experimentally.

In our study, we further found that the concentration of albumen LSM correlated positively with the concentration of albumen T while negatively with the concentration of albumen P5. No relationship was found between albumen steroids and OVOTR. Albumen LSM may act as an antibacterial protection to the developing embryo (Sellier et al., 2007; Guyot et al., 2016; Javurkova et al., 2019; Svobodova et al., 2019), and also as a stimulator of innate immunity (Saino et al., 2002; Bonisoli-Alquati et al., 2010; Bedrani et al., 2013). Similarly, yolk T has been shown to play a beneficial role in accelerating the growth of avian embryos (Groothuis and Schwabl, 2008; Navara and Mendonca, 2008; Ruuskanen, 2015). Thus, it is possible that the positive association between albumen LSM and T may be the result of passive allocation mechanism of quality females to produce quality eggs with maternally derived compounds and to achieve optimal phenotype and fitness of progeny. On the other hand, a growth-regulatory effect has also been found for albumen LSM, where especially in precocial species, experimentally increased albumen LSM resulted in a reduced tarsus length in quail chicks (Javurkova et al., 2015) and scaled body mass in ducklings (Svobodova et al., 2021). Given the lack of studies demonstrating the effect of albumen steroids on the avian embryo, we can only hypothesize that if we consider the growth-reducing effect of albumen LSM on the embryo, then higher concentrations of albumen T might be a compensatory compound that may favour growth in embryos exposed to higher concentrations of growth-reducing albumen LSM. Another possible assumption for the interactive relationship between T and P5 deposition in the albumen of eggs with higher albumen LSM concentrations could be related to the fact that P5 is a precursor for the synthesis of androgens and other steroids (Payne and Hales, 2004). Thus, it is likely that the lower concentration of P5 in the albumen of eggs with higher concentrations of T, whose increased deposition into the egg yolk caused oxidative stress (Giraudeau and Ducatez, 2016; Parolini et al., 2017, 2018; Possenti et al., 2018), could be the result of preventing further synthesis of pro-oxidant androgenic steroids in eggs with higher concentrations of albumen T. However, all of these assumption needs further experimental verification. In any case, both in our study and in other recent studies, P5, along with P4, is the most abundant steroid in bird eggs (Merrill et al., 2019; Mouton et al., 2022) and this is also true for the eggs of other vertebrates such as frogs (Paitz and Dugas, 2022). On the other hand, there is also evidence that natural concentrations not of steroids but of other substances in the egg (yolk), such as fatty acids, play a primary role in influencing the developing embryo and resulted offspring phenotype (Mentesana et al., 2021). Thus, the physiological role of albumen P5 as a precursor of all other important steroids is difficult to grasp, and the association with albumen LSM and other albumen steroids such as A4 or 17-OHP5 observed in our study points to the need to further investigate the function and relationships between steroids and other compounds in the albumen and their effect on the avian embryo in future studies.

Higher deposition of PROTO IX into the eggshell is thought to provide surface antimicrobial protection of eggs (Ishikawa et al., 2010; but see Dearborn et al., 2017). In addition, in our previous study, we found that differently pigmented chicken eggs differ in albumen AMPs concentration (Javurkova et al., 2019). Therefore, we hypothesized that differently pigmented chicken eggs might also differ in the concentration of other maternally derived albumen substances such as steroids. In addition, previous studies have found an association between intensity of PROTO IX eggshell spotting, egg yolk antioxidants, and egg quality improved hatchlings prosperity and phenotype (Hargitai et al., 2016, 2018; Corti et al., 2018; Soler et al., 2018). In our study, we found that of the five maternal protein steroid hormones, only the concentration of albumen 17-OHP5 varied with both chicken breed and eggshell colour, which reflected the concentration of PROTO IX in the eggshell cuticle. Tinted and white eggs with low eggshell cuticle PROTO IX content had the highest concentrations of albumen 17-OHP5. However, concentrations of albumen 17-OHP5 in similarly coloured eggs differed also with respect to the chicken breed. These findings suggest that the deposition of PROTO IX into the eggshell structures is strongly genetically encoded, which has been extensively studied and demonstrated especially in poultry (Zheng et al., 2014; Samiullah et al., 2017; Wilson et al., 2017; Chen et al., 2021; Lu et al., 2021). However, in both wild birds and commercially reared chickens or Japanese quail (*Coturnix japonica*), the hormonally-mediated effects of female condition (e.g. age or redox potential) and environmental conditions (e.g. diet and temperature) on intra-specific differences in PROTO IX deposition into the eggshell have been identified (Gosler et al., 2011; Ebeid et al., 2012; Duval et al., 2014). For example, dietary $Ca^{2+}$ deficiency leading to eggshell thinning has been shown to lead to increased deposition of PROTO IX in the eggshell due to increased progesterone production by the female (Soh and Koga, 1997). This is because P4 is responsible for the synthesis of calbindin, a protein that binds Ca2+ and thus reduces its transport to the shell gland where it is deficient (Ebeid et al., 2012). On the other hand, elevated P4 levels lead to the accumulation of PROTO IX in the shell gland and their higher deposition into the shell, thus compensating for its thinness (Soh and Koga, 1997; Gosler et al., 2011). Thus, it seems that it is primarily P4 that is one of the major regulatory mechanisms acting on the observed intra-specific variability of PROTO IX eggshell pigmentation in birds. In our study, however, we found an association only between the concentration of albumen 17-OHP5 and PROTO IX in the eggshell cuticle. This may be due to the fact that protein steroid concentrations may not fully reflect the hormonal status of the female at the time of egg formation. Moreover, while in songbirds most of PROTO IX is deposited in the eggshell cuticle or uppermost layers in the form of spotting, where it may perform, for example, a shell-strengthening or signalization functions (Gosler et al., 2005), in chickens this is not the case, and most PROTO IX has been found not in the eggshell cuticle but in the lower eggshell calcified layers (Samiullah and Roberts, 2014; Samiullah et al., 2015), where PROTO IX has not been analysed in our study. Thus, it is very likely that because the differently coloured chicken eggs are strongly genetically encoded (Li et al., 2006, 2016), there is little room for any physiological imprinting by the female that would be reflected in both the amount of PROTO IX deposited into the eggshell cuticle and maternally deposited steroids into the albumen.

Moreover, in our dataset, we used only 1–2 eggs produced by different females of a given breed. This is not sufficient to capture the effect of female condition or hormonally mediated influence of extrinsic factors such as diet or rearing and environmental conditions, which may be represented by the concentrations of steroids in albumen, on the variability of PROTO IX pigmentation.

In conclusion, our correlation study is the first to highlight possible synergistic and antagonistic relationships between maternally derived albumen steroids, proteins and eggshell PROTO IX pigmentation, which are likely the result of both passive and compensatory allocation mechanisms. Although, in the absence of studies, we can only speculate on the role of these albumen steroids as passive or compensatory maternal effects, one might argue whether it is even possible for the albumen hormones and to have any effect on the developing embryo. However, in reality there is no physical barrier between the yolk and the albumen as the perivitelline membrane surrounding the yolk degrades and disappears within the first few days of incubation (Jensen, 1969). Furthermore, many experimental studies investigating the fate of hormones in the egg and their metabolism have successfully injected labelled androgens directly into the albumen instead of the yolk and have shown that embryo/steroid interactions do occur (Paitz et al., 2011; Paitz and Casto, 2012; Paitz and Cagney, 2019). Moreover, given recent studies showing that the effect of hormones on the embryo is most likely through the extraembryonic membranes (Kumar et al., 2019) and that these are in close contact with the adjacent albumen, we believe that the effect of hormones and their relationship to other essential compounds contained in both the yolk and albumen (Valcu et al., 2019; Mentesana et al., 2021) and their effect on the embryo should no longer be overlooked. Instead, future studies focusing on the effect of egg maternal compounds on the developing embryo should analyse substances in both the albumen and yolk to obtain a complete biochemical profile of the whole egg, which is already being done in recent studies (Paitz et al., 2011; Paitz and Cagney, 2019; Paitz and Dugas, 2022). This comprehensive screening of egg maternal compounds should reveal correlations between the concentrations of individual compounds and serve as a guide for future experimental studies. These studies should then focus on more complex manipulation of multiple egg maternal compounds and consider whether they are in a synergistic or antagonistic relationship. In addition, experimental studies should also focus on monitoring post-treatment levels of egg maternal compounds and their metabolic fate *in ovo* to better determine the complex nature and interactions within egg maternal compounds and the embryo.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

The dataset generated and analysed in this study is available on Figshare https://figshare.com/ under this link https://doi.org/10.6084/m9.figshare.20559096.v1

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*Author contributions*

**Veronika Gvoždíková Javůrková:** Conceptualization, Investigation, Data curation, Supervision, Software, Visualisation, Validation, Funding acquisition, Writing – original draft. **Ivan Mikšík:** Methodology, Validation, Writing – review & editing.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2023.111401.

## References

Badyaev, A., Acevedo Seaman, D., Navara, K., Hill, G., Mendonca, M., 2006. Evolution of sex-biased maternal effects in birds: III. Adjustment of ovulation order can enable sex-specific allocation of hormones, carotenoids, and vitamins. J. Evol. Biol. 19, 1044–1057.

Baron, F., Jan, S., Gonnet, F., Pasco, M., Jardin, J., Giudici, B., Gautier, M., Guérin-Dubiard, C., Nau, F., 2014. Ovotransferrin plays a major role in the strong bactericidal effect of egg white against the *Bacillus cereus* group. J. Food Prot. 77, 955–962. https://doi.org/10.4315/0362-028x.jfp-13-473.

Bates, D., Machler, M., Bolker, B.M., Walker, S.C., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–48.

Bebbington, K., Groothuis, T.G.G., 2021. Who listens to mother? A whole-family perspective on the evolution of maternal hormone allocation. Biol. Rev. 96, 1951–1968. https://doi.org/10.1111/brv.12733.

Bedrani, L., Helloin, E., Guyot, N., Rehault-Godbert, S., Nys, Y., 2013. 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, 1–10. https://doi.org/10.1186/1471-2180-13-128.

Belnap, S.C., Currea, J.P., Lickliter, R., 2019. Prenatal incubation temperature affects neonatal precocial birds' locomotor behavior. Physiol. Behav. 206, 51–58. https://doi.org/10.1016/j.physbeh.2019.03.002.

Bonisoli-Alquati, A., Rubolini, D., Romano, M., Cucco, M., Fasola, M., Caprioli, M., Saino, N., 2010. Egg antimicrobials, embryo sex and chick phenotype in the yellow-legged gull. Behav. Ecol. Sociobiol. 64, 845–855. https://doi.org/10.1007/s00265-010-0901-8.

Caulfield, M.P., Padula, M.P., 2020. HPLC MS-MS analysis shows measurement of corticosterone in egg albumen is not a valid indicator of chicken welfare. Animals 10 (821), 1–10. https://doi.org/10.3390/ani10050821.

Chen, X., He, Z., Li, X., Song, J., Huang, M., Shi, X., Li, X., Li, J., Xu, G., Zheng, J., 2021. Cuticle deposition duration in the uterus is correlated with eggshell cuticle quality in White Leghorn laying hens. Sci. Rep. 11, 22100. https://doi.org/10.1038/s41598-021-01718-0.

Cook, M.I., Beissinger, S.R., Toranzos, G.A., Rodriguez, R.A., Arendt, W.J., 2003. Trans-shell infection by pathogenic micro-organisms reduces the shelf life of non-incubated bird's eggs: A constraint on the onset of incubation? Proc. R. Soc. B Biol. Sci. 270, 2233–2240. https://doi.org/10.1098/rspb.2003.2508.

Cook, M.I., Beissinger, S.R., Toranzos, G.A., Rodriguez, R.A., Arendt, W.J., 2005. Microbial infection affects egg viability and incubation behavior in a tropical passerine. Behav. Ecol. 16, 30–36. https://doi.org/10.1093/beheco/arh131.

Corti, M., Romano, A., Costanzo, A., Bentz, A.B., Navara, K.J., Parolini, M., Saino, N., Rubolini, D., 2018. Protoporphyrin-based eggshell pigmentation predicts hatching success and offspring sex ratio in the barn swallow. J. Avian Biol. 49 (e01642), 1–17. https://doi.org/10.1111/jav.01642.

D'Alba, L., Torres, R., Waterhouse, G.I.N., Eliason, C., Hauber, M.E., Shawkey, M.D., 2017. What does the eggshell cuticle do? A functional comparison of avian eggshell cuticles. Physiol. Biochem. Zool. 90, 588–599. https://doi.org/10.1086/693434.

De Baere, S., Larsen, T.R., Devreese, M., De Backer, P., De Neve, L., Fairhurst, G., Lens, L., Croubels, S., 2015. Use of LC-MS-MS as an alternative to currently available immunoassay methods to quantitate corticosterone in egg yolk and albumen. Anal. Bioanal. Chem. 407, 4351–4362. https://doi.org/10.1007/s00216-014-8269-7.

Dearborn, D.C., Page, S.M., Dainson, M., Hauber, M.E., Hanley, D., 2017. Eggshells as hosts of bacterial communities: An experimental test of the antimicrobial egg coloration hypothesis. Ecol. Evol. 7, 9711–9719. https://doi.org/10.1002/ece3.3508.

Downing, J.A., Bryden, W.L., 2008. Determination of corticosterone concentrations in egg albumen: A non-invasive indicator of stress in laying hens. Physiol. Behav. 95, 381–387. https://doi.org/10.1016/j.physbeh.2008.07.001.

Duval, C., Cassey, P., Mikšík, I., Reynolds, S.J., Spencer, K.A., 2014. Condition-dependent strategies of eggshell pigmentation: an experimental study of Japanese quail (*Coturnix coturnix japonica*). J. Exper. Bio. 216 (4), 700–708.

Ebeid, T.A., Suzuki, T., Sugiyama, T., 2012. High ambient temperature influences eggshell quality and calbindin-D28k localization of eggshell gland and all intestinal segments of laying hens. Poult. Sci. 91 (9), 2282–2287. https://doi.org/10.3382/ps.2011-01898.

Giansanti, F., Massucci, M.T., Giardi, M.F., Nozza, F., Pulsinelli, E., Nicolini, C., Botti, D., Antonini, G., 2005. Antiviral activity of ovotransferrin derived peptides. Biochem. Biophysic. Res. Comm. 331, 69–73. https://doi.org/10.1016/j.bbrc.2005.03.125.

Gil, D., 2003. Golden eggs: Maternal manipulation of offspring phenotype by egg androgen in birds. Ardeola 50, 281–294.

Giraudeau, M., Ducatez, S., 2016. Co-adjustment of yolk antioxidants and androgens in birds. Biol. Lett. 12 https://doi.org/10.1098/rsbl.2016.0676, 20160676.

Giraudeau, M., Ziegler, A.K., Pick, J.L., Ducatez, S., Canale, C.I., Tschirren, B., 2017. Interactive effects of yolk testosterone and carotenoid on prenatal growth and offspring physiology in a precocial bird. Behav. Ecol. 28, 31–38. https://doi.org/10.1093/beheco/arw127.

Gosler, A.G., Higham, J.P., Reynolds, S.J., 2005. Why are birds' eggs speckled? Ecol. Lett. 8, 1105–1113. https://doi.org/10.1111/j.1461-0248.2005.00816.x.

Gosler, A.G., Connor, O.R., Bonser, R.H.C., 2011. Protoporphyrin and eggshell strength: preliminary findings from a passerine bird. Avian Biol. Res. 4 (4), 214–223.

Goth, A., Eising, C.M., Herberstein, M.E., Groothuis, T.G.G., 2008. Consistent variation in yolk androgens in the australian brush-turkey, a species without sibling competition or parental care. Gen. Comp. Endocrinol. 155, 742–748. https://doi.org/10.1016/j.ygcen.2007.11.004.

Grizard, S., Dini-Andreote, F., Tieleman, B.I., Salles, J.F., 2014. Dynamics of bacterial and fungal communities associated with eggshells during incubation. Ecol. Evol. 4, 1140–1157. https://doi.org/10.1002/ece3.1011.

Grizard, S., Versteegh, M.A., Ndithia, H.K., Salles, J.F., Tieleman, B.I., 2015. Shifts in bacterial communities of eggshells and antimicrobial activities in eggs during incubation in a ground-nesting passerine. PLoS One 10 (4), e0121716. https://doi.org/10.1371/journal.pone.0121716.

Groothuis, T.G.G., Schwabl, H., 2008. Hormone-mediated maternal effects in birds: Mechanisms matter but what do we know of them? Phil. Trans. R. Soc. B. Biol. Sci. 363, 1647–1661. https://doi.org/10.1098/rstb.2007.2087.

Groothuis, T.G.G., Muller, W., von Engelhardt, N., Carere, C., Eising, C., 2005. Maternal hormones as a tool to adjust offspring phenotype in avian species. Neurosci. Biobehav. Rev. 29, 329–352. https://doi.org/10.1016/j.neubiorev.2004.12.002.

Groothuis, T.G.G., Eising, C.M., Blount, J.D., Surai, P., Apanius, V., Dijkstra, C., Müller, W., 2006. Multiple pathways of maternal effects in black-headed gull eggs: constraint and adaptive compensatory adjustment. J. Evol. Biol. 19, 1304–1313. https://doi.org/10.1111/j.1420-9101.2005.01072.x.

Guyot, N., Rehault-Godbert, S., Slugocki, C., Harichaux, G., Labas, V., Helloin, E., Nys, Y., 2016. Characterization of egg white antibacterial properties during the first half of incubation: A comparative study between embryonated and unfertilized eggs. Poult. Sci. 95, 2956–2970. https://doi.org/10.3382/ps/pew271.

Hargitai, R., Nagy, G., Herenyi, M., Nyiri, Z., Laczi, M., Hegyi, G., Eke, Z., Török, J., 2016. Darker eggshell spotting indicates lower yolk antioxidant level and poorer female quality in the Eurasian great tit (*Parus major*). Auk 133, 131–146. https://doi.org/10.1642/auk-15-128.1.

Hargitai, R., Herenyi, M., Nagy, G., Torok, J., 2018. Eggshell spotting pattern is related to hatching asynchrony, hematocrit value and growth of nestling great tits *Parus major*. J. Avian Biol. 49, e01827 https://doi.org/10.1111/jav.01827.

Harrell, Jr.F., Dupont, C.H., 2019. Hmisc: Harrell Miscellaneous. R Package Version 4.2-0. https://CRAN.R-project.org/package=Hmisc.

Hegyi, G., Herenyi, M., Szollosi, E., Rosivall, B., Torok, J., Groothuis, T.G.G., 2011. Yolk androstenedione, but not testosterone, predicts offspring fate and reflects parental quality. Behav. Ecol. 22, 29–38. https://doi.org/10.1093/beheco/arq165.

Horrocks, N.P.C., Tieleman, B.I., Matson, K.D., 2011. A simple assay for measurement of ovotransferrin - a marker of inflammation and infection in birds. Methods Ecol. Evol. 2, 518–526. https://doi.org/10.1111/j.2041-210X.2011.00096.x.

Hsu, B.-Y., Dijkstra, C., Groothuis, T.G.G., 2016. No escape from mother's will: Effects of maternal testosterone on offspring reproductive behaviour far into adulthood. Anim. Behav. 117, 135–144. https://doi.org/10.1016/j.anbehav.2016.05.004.

Hsu, B.-Y., Dijkstra, C., Darras, V.M., de Vries, B., Groothuis, T.G.G., 2017. Maternal thyroid hormones enhance hatching success but decrease nestling body mass in the rock pigeon (*Columba livia*). Gen. Comp. Endocrinol. 240, 174–181. https://doi.org/10.1016/j.ygcen.2016.10.011.

Hsu, B.-Y., Pakanen, V.-M., Boner, W., Doligez, B., Eeva, T., Groothuis, T.G.G., Korpimäki, E., Laaksonen, T., Lelono, A., Monaghan, P., Sarraude, T., Thomson, R.L., Tolvanen, J., Tschirren, B., Vásquez, R.A., Ruuskanen, S., 2022. Maternally transferred thyroid hormones and life-history variation in birds. J. Anim. Ecol. 91, 1489–1506. https://doi.org/10.1111/1365-2656.13708.

Huopalahti, R., Anton, M., López-Fandiño, R., Schade, R., 2007. Bioactive Egg Compounds, 1st ed. Springer, Berlin, Heidelberg.

Ibrahim, H.R., Sugimoto, Y., Aoki, T., 2000. Ovotransferrin antimicrobial peptide (otap-92) kills bacteria through a membrane damage mechanism. Biochim. Et Biophys. Acta Gen. Subj. 1523, 196–205. https://doi.org/10.1016/s0304-4165(00)00122-7.

Ibrahim, H.R., Hoq, M.I., Aoki, T., 2007. Ovotransferrin possesses sod-like superoxide anion scavenging activity that is promoted by copper and manganese binding. Int. J. Biol. Macromol. 41, 631–640. https://doi.org/10.1016/j.ijbiomac.2007.08.005.

Ishikawa, S., Suzuki, K., Fukuda, E., Arihara, K., Yamamoto, Y., Mukai, T., Itoh, M., 2010. Photodynamic antimicrobial activity of avian eggshell pigments. FEBS Lett. 584, 770–774. https://doi.org/10.1016/j.febslet.2009.12.041.

Janczak, A.M., Torjesen, P., Rettenbacher, S., 2009. Environmental effects on steroid hormone concentrations in laying hens' eggs. Acta Agricult. Scand. Sect. A Anim. Sci. 59, 80–84. https://doi.org/10.1080/09064700903023348.

Javurkova, V., Albrecht, T., Mrazek, J., Kreisinger, J., 2014. Effect of intermittent incubation and clutch covering on the probability of bacterial trans-shell infection. Ibis 156, 374–386. https://doi.org/10.1111/ibi.12126.

Javurkova, V., Krkavcova, E., Kreisinger, J., Hyrsl, P., Hyankova, L., 2015. Effects of experimentally increased in ovo lysozyme on egg hatchability, chicks complement activity, and phenotype in a precocial bird. J. Exp. Zool. Part A-Ecol. Genet. Physiol. 323, 497–505. https://doi.org/10.1002/jez.1935.

Javurkova, V.G., Pokorna, M., Miksik, I., Tumova, E., 2019. Concentration of egg white antimicrobial and immunomodulatory proteins is related to eggshell pigmentation across traditional chicken breeds. Poult. Sci. 98, 6931–6941. https://doi.org/10.3382/ps/pez472.

Jensen, C., 1969. Ultrastructural changes in the avian vitelline membrane during embryonic development. Development 21, 467–484.

Kennedy, G.Y., Vevers, H.G., 1976. Survey of avian eggshell pigments. Comp. Biochem. Physiol. B-Biochem. & Mol. Biol. 55, 117–123. https://doi.org/10.1016/0305-0491(76)90183-8.

Kim, J., Choi, Y.-H., 2014. Differential abundance of egg white proteins in laying hens treated with corticosterone. J. Agric. Food Chem. 62, 12346–12359.

Korner-Nievergelt, F., Roth, T., von Felten, S., Guélat, J., Almasi, B., Korner-Nievergelt, P., 2015. Bayesian data analysis in ecology using linear models with R, BUGS, and Stan, 1st ed. Academic Press, Elsevier.

Kozlowski, C.P., Ricklefs, R.E., 2010. Egg size and yolk steroids vary across the laying order in cockatiel clutches: A strategy for reinforcing brood hierarchies? Gen. Comp. Endocrinol. 168, 460–465. https://doi.org/10.1016/j.ygcen.2010.06.006.

Krist, M., 2011. Egg size and offspring quality: A meta-analysis in birds. Biol. Rev. 86, 692–716. https://doi.org/10.1111/j.1469-185X.2010.00166.x.

Kuettner, K.E., Soble, L.W., Ray, R.D., Croxen, R.L., Passovoy, M., Eisenstein, R., 1970. Lysozyme in epiphyseal cartilage. 2. Effect of egg white lysozyme on mouse embryonic femurs in organ cultures. J. Cell Biol. 44, 329–335. https://doi.org/10.1083/jcb.44.2.329.

Kumar, N., Lohrentz, A., Gahr, M., Groothuis, T.G.G., 2019. Steroid receptors and their regulation in avian extraembryonic membranes provide a novel substrate for hormone mediated maternal effects. Sci. Rep. 9, 11501. https://doi.org/10.1038/s41598-019-48001-x.

Le Bouc, Y., Groyer, A., Cadepond, F., Groyer-Schweizer, G., Robel, P., Baulieu, E.E., 1985. 1985 Effects of progesterone and tamoxifen on glucocorticosteroid-induced egg-white protein synthesis in the chick oviduct. Endocrinology. 116, 2384–2392.

Li, X.Y., Xu, G.Y., Hou, Z.C., Zhao, R., Yang, N., 2006. Variation of eggshell colour in different egg-type chickens. Arch. Fur Geflugelkunde 70, 278–282.

Li, G.Q., Sun, C.J., Wu, G.Q., Shi, F.Y., Liu, A.Q., Yang, N., 2016. ITRAq-based quantitative proteomics identifies potential regulatory proteins involved in chicken eggshell brownness. PLoS One 11, e0168750. https://doi.org/10.1371/journal.pone.0168750.

Lu, M.-Y., Xu, L., Qi, G.-H., Zhang, H.-J., Qiu, K., Wang, J., Wu, S.-G., 2021. Mechanisms associated with the depigmentation of brown eggshells: a review. Poult. Sci. 100 (8), 101273 https://doi.org/10.1016/j.psj.2021.101273.

Matsunaga, M., Ukena, K., Tsutsui, K., 2001. Expression and localization of cytochrome p450 17 alpha-hydroxylase/c17,20-lyase in the avian brain. Brain Res. 899, 112–122. https://doi.org/10.1016/s0006-8993(01)02217-x.

Matsunaga, M., Ukena, K., Tsutsui, K., 2002. Androgen biosynthesis in the quail brain. Brain Res. 948, 180–185. https://doi.org/10.1016/s0006-8993(02)03147-5.

Mentesana, L., Andersson, M.N., Casagrande, S., Goymann, W., Isaksson, C., Hau, M., 2021. Natural variation in yolk fatty acids, but not androgens, predicts offspring fitness in a wild bird. Front. Zool. 18 (1), 1–12.

Merrill, L., Chiavacci, S.J., Paitz, R.T., Benson, T.J., 2019. Quantification of 27 yolk steroid hormones in seven shrubland bird species: Interspecific patterns of hormone deposition and links to life history, development, and predation risk. Can. J. Zool. 97, 1–12. https://doi.org/10.1139/cjz-2017-0351.

Miksik, I., Holan, V., Deyl, Z., 1996. Avian eggshell pigments and their variability. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 113, 607–612. https://doi.org/10.1016/0305-0491(95)02073-x.

Mouton, J.C., Duckworth, R.A., 2021. Maternally derived hormones, neurosteroids and the development of behaviour. Proc. R. Soc. B Biol. Sci. 288, 20202467. https://doi.org/10.1098/rspb.2020.2467.

Mouton, J.C., Duckworth, R.A., Paitz, R.T., Martin, T.E., 2022. Nest predation risk and deposition of yolk steroids in a cavity-nesting songbird: An experimental test. J. Exp. Biol. 225, jeb243047. https://doi.org/10.1242/jeb.243047.

Mundry, R., Nunn, C.L., 2009. Stepwise model fitting and statistical inference: turning noise into signal pollution. Am. Nat. 173, 119–123.

Narushin, V.G., 2005. Production, modeling, and education - egg geometry calculation using the measurements of length and breadth. Poult. Sci. 84, 482–484. https://doi.org/10.1093/ps/84.3.482.

Navara, K.J., Mendonca, M.T., 2008. Yolk androgens as pleiotropic mediators of physiological processes: A mechanistic review. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 150, 378–386. https://doi.org/10.1016/j.cbpa.2008.05.002.

Navara, K.J., Badyaev, A.V., Mendonca, M.T., Hill, G.E., 2006. Yolk antioxidants vary with male attractiveness and female condition in the house finch (*Carpodacus mexicanus*). Physiol. Biochem. Zool. 79, 1098–1105. https://doi.org/10.1086/507661.

Ogura, Y., Haraguchi, S., Nagino, K., Ishikawa, K., Fukahori, Y., Tsutsui, K., 2016. 7 alpha-hydroxypregnenolone regulates diurnal changes in sexual behavior of male quail. Gen. Comp. Endocrinol. 227, 130–135. https://doi.org/10.1016/j.ygcen.2015.11.010.

Osserman, E.F., Lawlor, D.P., 1966. Serum and urinary lysozyme (Muramidase) in monocytic and monomyelocytic leukemia. J. Exp. Med. 124, 921–933. https://doi.org/10.1084/jem.124.5.921.

Paitz, R.T., Cagney, E., 2019. In ovo metabolism of progesterone to 5 beta-pregnanedione in chicken eggs: Implications for how yolk progesterone influences embryonic development. Gen. Comp. Endocrinol. 282, 113221.

Paitz, R.T., Casto, J.M., 2012. The decline in yolk progesterone concentrations during incubation is dependent on embryonic development in the European Starling. Gen. Comp. Endocrinol. 176, 415–419. https://doi.org/10.1016/j.ygcen.2011.12.014.

Paitz, R.T., Dugas, M.B., 2022. Steroid levels in frog eggs: Manipulations, developmental changes, and implications for maternal steroid effects. J. Exp. Zool. A Ecol. Integr. Physiol. 337, 293–302. https://doi.org/10.1002/jez.2566.

Paitz, R.T., Bowden, R.M., Casto, J.M., 2011. Embryonic modulation of maternal steroids in european starlings (*Sturnus vulgaris*). Proc. R. Soc. B Biol. Sci. 278, 99–106. https://doi.org/10.1098/rspb.2010.0813.

Parolini, M., Romano, A., Possenti, C.D., Caprioli, M., Rubolini, D., Saino, N., 2017. Contrasting effects of increased yolk testosterone content on development and oxidative status in gull embryos. J. Exp. Biol. 220, 625–633. https://doi.org/10.1242/jeb.145235.

Parolini, M., Possenti, C.D., Romano, A., Caprioli, M., Rubolini, D., Saino, N., 2018. Physiological increase of yolk testosterone level does not affect oxidative status and telomere length in gull hatchlings. PLoS One 13, e0206503. https://doi.org/10.1371/journal.pone.0206503.

Partecke, J., Hegyi, G., Fitze, P.S., Gasparini, J., Schwabl, H., 2020. Maternal effects and urbanization: Variation of yolk androgens and immunoglobulin in city and forest blackbirds. Ecol. Evol. 10, 2213–2224. https://doi.org/10.1002/ece3.6058.

Payne, A.H., Hales, D.B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. 25, 947–970. https://doi.org/10.1210/er.2003-0030.

Peralta-Sanchez, J.M., Martin-Vivaldi, M., Martin-Platero, A.M., Martinez-Bueno, M., Onate, M., Ruiz-Rodríguez, M., Soler, J.J., 2012. Avian life history traits influence eggshell bacterial loads: A comparative analysis. Ibis. 154, 725–737. https://doi.org/10.1111/j.1474-919X.2012.01256.x.

Peralta-Sanchez, J., Martin-Platero, A.M., Wegener-Parfrey, L., Martinez-Bueno, M., Rodriguez-Ruano, S., Navas-Molina, J.A., Vázquez-Baeza, Y., Martín-Gálvez, D., Martín-Vivaldi, M., Ibáñez-Álamo, J.D., Knight, R., Soler, J.J., 2018. Bacterial density rather than diversity correlates with hatching success across different avian species. FEMS Microbiol. Ecol. 94 https://doi.org/10.1093/femsec/fiy022 fiy022.

Poisbleau, M., Demongin, L., Angelier, F., Dano, S., Lacroix, A., Quillfeldt, P., 2009. What ecological factors can affect albumen corticosterone levels in the clutches of seabirds? Timing of breeding, disturbance and laying order in rockhopper penguins (*Eudyptes chrysocome chrysocome*). Gen. Comp. Endocrinol. 162, 139–145. https://doi.org/10.1016/j.ygcen.2009.03.022.

Possenti, C.D., Secomandi, S., Schiavon, A., Caprioli, M., Rubolini, D., Romano, A., Saino, N., Parolini, M., 2018. Independent and combined effects of egg pro- and anti-oxidants on gull chick phenotype. J. Exp. Biol. 221, jeb 174300. https://doi.org/10.1242/jeb.174300.

R Development Core Team, 2017. R: A Language & Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria http://www.R-project.org.

Royle, N.J., Surai, P.F., Hartley, I.R., 2001. Maternally derived androgens and antioxidants in bird eggs: Complementary but opposing effects? Behav. Ecol. 12, 381–385.

RStudioTeam, 2015. RStudio: integrated development for R. RStudio, Inc, Boston.

Rubolini, D., Romano, M., Navara, K.J., Karadas, F., Ambrosini, R., Caprioli, M., Saino, N., 2011. Maternal effects mediated by egg quality in the yellow-legged gull *Larus michahellis* in relation to laying order and embryo sex. Front. Zool. 8, 24.

Ruuskanen, S., 2015. Hormonally-mediated maternal effects in birds: Lessons from the flycatcher model system. Gen. Comp. Endocrinol. 224, 283–293.

Ruuskanen, S., Groothuis, T.G.G., Schaper, S.V., Darras, V.M., de Vries, B., Visser, M.E., 2016. Temperature-induced variation in yolk androgen and thyroid hormone levels in avian eggs. Gen. Comp. Endocrinol. 235, 29–37.

Ruuskanen, S., Groothuis, T.G.G., Darras, V.M., Gienapp, P., Schaper, S.V., Visser, M.E., 2017. Egg thyroid hormones: An unexplored mechanism for maternal effects in birds. Integr. Comp. Biol. 57. E393-E393.

Saino, N., Dall'ara, P., Martinelli, R., Moller, A.P., 2002. Early maternal effects and antibacterial immune factors in the eggs, nestlings and adults of the barn swallow. J. Evol. Biol. 15, 735–743. https://doi.org/10.1046/j.1420-9101.2002.00448.x.

Sakamoto, S., Sakamoto, M., Goldhaber, P., Glimcher, M.J., 1974. Inhibition of mouse bone collagenase by lysozyme. Calcif. Tissue Res. 14, 291–299. https://doi.org/10.1007/bf02060303.

Samiullah, S., Roberts, J.R., 2014. The eggshell cuticle of the laying hen. Worlds Poult. Sci. J. 70, 693–707. https://doi.org/10.1017/s0043933914000786.

Samiullah, S., Roberts, J.R., Chousalkar, K., 2015. Eggshell color in brown-egg laying hens - a review. Poult. Sci. 94, 2566–2575. https://doi.org/10.3382/ps/pev202.

Samiullah, S., Roberts, J., Wu, S.B.A., 2017. Downregulation of ALAS1 by nicarbazin treatment underlies the reduced synthesis of protoporphyrin IX in shell gland of laying hens. Sci. Rep. 7 (1), 6253. https://doi.org/10.1038/s41598-017-06527-y.

Sarraude, T., Hsu, B.Y., Groothuis, T.G.G., Ruuskanen, S., 2020. Testing different forms of regulation of yolk thyroid hormone transfer in pied flycatchers. J. Exp. Biol. 223 https://doi.org/10.1242/jeb.226688 jeb226688.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

Sellier, N., Vidal, M.L., Baron, F., Michel, J., Gautron, J., Protais, M., Beaumont, C., Gautier, M., Nys, Y., 2007. Estimations of repeatability and heritability of egg albumen antimicrobial activity and of lysozyme and ovotransferrin concentrations. Br. Poult. Sci. 48, 559–566. https://doi.org/10.1080/00071660701592367.

Soh, T., Koga, O., 1997. The effect of progesterone and estradiol-17 beta on the pigment accumulation of the shell gland in Japanese quail pretreated with aminoglutethimide. J. Fac. Agric. Kyushu Univ. 42, 147–150.

Soler, J.J., Ruiz-Castellano, C., Figuerola, J., Martinez-de la Puente, J., Ruiz-Rodriguez, M., Tomás, G., 2018. Egg colouration predicts brood size, telomere length and body condition of spotless starling fledglings. J. Avian Biol. 49, e01686 https://doi.org/10.1111/jav.01686.

Sosvorova, L., Vitku, J., Chlupacova, T., Mohapl, M., Hampl, R., 2015. Determination of seven selected neuro- and immunomodulatory steroids in human cerebrospinal fluid and plasma using LC-MS/MS. Steroids. 98, 1–8. https://doi.org/10.1016/j.steroids.2015.01.019.

Svobodova, J., Smidova, L., Gvozdikova Javurkova, V., 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. https://doi.org/10.1242/jeb.201442.

Svobodova, J., Kreisinger, J., Javurkova, V.G., 2021. Temperature-induced changes in egg white antimicrobial concentrations during pre-incubation do not influence bacterial trans-shell penetration but do affect hatchling phenotype in mallards. PeerJ. 9, e12401 https://doi.org/10.7717/peerj.12401.

Tsutsui, K., Haraguchi, S., Inoue, K., Miyabara, H., Suzuki, S., Ubuka, T., 2012. Control of circadian activity of birds by the interaction of melatonin with 7 alpha-hydroxypregnenolone, a newly discovered neurosteroid stimulating locomotion. J. Ornithol. 153, S235–S243. https://doi.org/10.1007/s10336-011-0795-x.

Tsutsui, K., Haraguchi, S., Hatori, M., Hirota, T., Fukada, Y., 2013a. Biosynthesis and biological actions of pineal neurosteroids in domestic birds. Neuroendocrinology. 98, 97–105. https://doi.org/10.1159/000353782.

Tsutsui, K., Haraguchi, S., Inoue, K., Miyabara, H., Ubuka, T., Hatori, M., Hirota, T., Fukada, Y., 2013b. New biosynthesis and biological actions of avian neurosteroids. J. Exp. Neurosci. 7, 15–29. https://doi.org/10.4137/jen.s11148.

Tsutsui, K., Haraguchi, S., Vaudry, H., 2018. 7 alpha-hydroxypregnenolone regulating locomotor behavior identified in the brain and pineal gland across vertebrates. Gen. Comp. Endocrinol. 265, 97–105. https://doi.org/10.1016/j.ygcen.2017.09.014.

Valcu, C.-M., Scheltema, R.A., Schweiggert, R.M., Valcu, M., Teltscher, K., Walther, D.M., Carle, R., Kempenaers, B., 2019. Life history shapes variation in egg composition in the blue tit *Cyanistes caeruleus*. Commun. Biol. 2 (6), 1–14.

von Engelhardt, N., Groothuis, T.G.G., 2011. Maternal hormones in avian eggs. In: Norris, D.O., Lopez, K.H. (Eds.), Hormones and Reproduction of Vertebrates. Academic Press, Cambridge, pp. 91–127.

Wei, T., Simko, V., 2021. R package 'corrplot': Visualization of a Correlation Matrix (version 0.92). Available from: https://github.com/taiyun/corrplot.

Wellman-Labadie, O., Picman, J., Hincke, M.T., 2008a. Antimicrobial activity of cuticle and outer eggshell protein extracts from three species of domestic birds. Br. Poult. Sci. 49, 133–143. https://doi.org/10.1080/00071660802001722.

Wellman-Labadie, O., Picman, J., Hincke, M.T., 2008b. Comparative antibacterial activity of avian egg white protein extracts. Br. Poult. Sci. 49, 125–132. https://doi.org/10.1080/00071660801938825.

Weng, J.H., Chung, B.C., 2016. Nongenomic actions of neurosteroid pregnenolone and its metabolites. Steroids. 111, 54–59. https://doi.org/10.1016/j.steroids.2016.01.017.

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

Williams, T.D., 1994. Intraspecific variation in egg size and egg composition in birds - effects on offspring fitness. Biol. Rev. Camb. Philos. Soc. 69, 35–59. https://doi.org/10.1111/j.1469-185X.1994.tb01485.x.

Williams, T.D., Groothuis, T.G.G., 2015. Egg quality, embryonic development, and post-hatching phenotype: an integrated perspective. In: Deeming, D.C., Reynolds, S.J. (Eds.), Nests, Eggs, and Incubation: New ideas about avian reproduction. Oxford University Press, London, pp. 113–126.

Wilson, P.W., Suther, C.S., Bain, M.M., Icken, W., Jones, A., Quinlan-Pluck, F., Olori, V., Gautron, J., Dunn, I.C., 2017. Understanding avian egg cuticle formation in the oviduct: a study of its origin and deposition. Biol. Reprod. 97, 39–49. https://doi.org/10.1093/biolre/iox070.

Wingfield, J.C., Wacker, D.W., Bentley, G.E., Tsutsui, K., 2018. Brain-derived steroids, behavior and endocrine conflicts across life history stages in birds: A perspective. Front. Endocrinol. 9 https://doi.org/10.3389/fendo.2018.00270.

Wu, J.P., Acero-Lopez, A., 2012. Ovotransferrin: Structure, bioactivities, and preparation. Food Res. Int. 46, 480–487. https://doi.org/10.1016/j.foodres.2011.07.012.

Wu, Q.F., Liu, H.H., Yang, Q.L., Wei, B., Wang, L.Y., Tang, Q., Wang, J., Xi, Y., Han, Ch., Wang, J., Li, L., 2022. Developmental transcriptome profiling of the tibial reveals the underlying molecular basis for why newly hatched quails can walk while newly hatched pigeons cannot. Front. Cell Develop. Biol. 10 https://doi.org/10.3389/fcell.2022.745129.

Xie, H., Huff, G.R., Huff, W.E., Balog, J.M., Rath, N.C., 2002. Effects of ovotransferrin on chicken macrophages and heterophil-granulocytes. Dev. Comp. Immun. 26, 805–815. https://doi.org/10.1016/s0145-305x(02)00028-9.

Yamanishi, H., Iyama, S., Yamaguchi, Y., Kanakura, Y., Iwatani, Y., 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, J., Mao, Z., Wang, X., Zhuang, J., Gong, S., Gao, Z., Xu, G., Yang, N., Sun, C., 2022. Identification of crucial genes and metabolites regulating the eggshell brownness in chicken. BMC Genomics 23, 761.

Zheng, Ch., Li, Z., Yang, N., Ning, Z., 2014. Quantitative expression of candidate genes affecting eggshell color. Anim. Sci. J. 85, 506–510.