The cuticle modulates ultraviolet reflectance of avian eggshells

Daphne C. Fecheyr-Lippens1,*, Branislav Igic1, Liliana D’Alba1, Daniel Hanley2, Aida Verdes3, Mande Holford3, Geoffrey I. N. Waterhouse4, Tomas Grim2, Mark E. Hauber5 and Matthew D. Shawkey1

ABSTRACT

Avian eggshells are variably coloured, yet only two pigments, biliverdin and protoporphyrin IX, are known to contribute to the dramatic diversity of their colours. By contrast, the contributions of structural or other chemical components of the eggshell are poorly understood. For example, unpigmented eggshells, which appear white to the human eye, vary in their ultraviolet (UV) reflectance, which may be detectable by birds. We investigated the proximate mechanisms for the variation in UV-reflectance of unpigmented bird eggshells using spectrophotometry, electron microscopy, chemical analyses, and experimental manipulations. We specifically tested how UV-reflectance is affected by the eggshell cuticle, the outermost layer of most avian eggshells. The chemical dissolution of the outer eggshell layers, including the cuticle, increased UV-reflectance for only eggshells that contained a cuticle. Our findings demonstrate that the outer eggshell layers, including the cuticle, absorb UV-light, probably because they contain higher levels of organic components and other chemicals, such as calcium phosphates, compared to the predominantly calcite-based eggshell matrix. These data highlight the need to examine factors other than the known pigments in studies of avian eggshell colour.

KEY WORDS: Avian eggshells, Cuticle, Light modulation, Ultraviolet reflectance, Biomimicry

INTRODUCTION

Understanding the proximate causes of variation in morphological traits like colour is critical to understanding their functions and evolution (Hill and McGraw, 2006). Eggshell coloration may serve several roles, including camouflage (Merilaia and Lind, 2005), sexual selection (Moreno and Osorno, 2003), or host-parasite egg mimicry and rejection (Yang et al., 2013). A recent study further suggested that colour produced by pigments modulates the amount of beneficial vs. harmful UV-light reaching the embryo by acting as an absorbing barrier (Maurer et al., 2015). However, many eggshells lack pigmentation (Hauber, 2014) and the mechanism by which they attenuate ultraviolet light is unknown (Kilner, 2006). Studying the proximate basis of egg coloration may also help provide inspiration for applied systems, including the development of biomimetic materials by identifying important factors that contribute to light modulation (Yoo et al., 2009; Li et al., 2010). Colours in nature can be produced by pigments, nanostructured architectures (generating structural colour), or a combination of both (Parker, 2000; Sun et al., 2013). Whereas pigments produce colour through the absorbance of light at specific wavelengths, structural colours are produced by selective reflectance, scattering or diffraction of light by nanostructured biological materials (Kinoshita et al., 2008; Srinivasarao, 1999).

Little is known about the mechanisms that generate eggshell coloration. Currently, only two classes of tetrapyrrole pigments (biliverdin and protoporphyrin IX) are considered to influence eggshell coloration of most bird species (Kennedy and Vevers, 1976). However, recent studies have shown that eggshell coloration of a number of different species cannot be explained solely by variation in biliverdin and protoporphyrin concentrations (Cassey et al., 2012a; Igic et al., 2012), suggesting that other mechanisms may contribute to the appearance of eggshells. Indeed, in addition to the two tetrapyrrole pigments avian eggshells consist of numerous other compounds that may selectively absorb light or modify the absorption properties of the two pigments.

In addition to pigments, eggshell proteins or nanostructures could contribute to eggshell coloration by either selectively absorbing certain wavelengths or enhancing light reflectance, respectively. Eggshells consists of about 4% organic and 96% inorganic material, the latter of which 98% is calcium carbonate, and the remainder includes calcium phosphates and metal ions (Hamilton, 1986). Furthermore, the external eggshell surface of most avian species is covered by a cuticle, a non-crystalized layer that can vary in thickness and consist of proteins, polysaccharides, lipids, calcium carbonate, and calcium phosphates (Kusuda et al., 2011; Mikhailov, 1997; Wedral et al., 1974). Aromatic amino acids of proteins (Holiday, 1936) and calcium phosphates (Bogrekci and Lee, 2004; Holzmann et al., 2009) also have distinctive absorption spectra compared to calcite and the two tetrapyrrole pigments. Both groups of molecules absorb maximally in the (near) UV-range, and are common constituents of eggshells (Hincke et al., 1992; Sparks, 1994). Moreover, the nanostructural organisation of calcium carbonate can produce structural colour [e.g. nacre (Griségo, 1957; Bonderer et al., 2008; Finnemore, 2012)]. Critically, the eggshell cuticle differs both in composition and structure from the underlying crystalized eggshell (Baker and Balch, 1962; Kusuda et al., 2011) and therefore may differentially affect light modulation. Indeed, it has been shown that an extremely smooth cuticle produces glossiness and iridescence in tinamou eggs (Igic et al., 2015).

Here, we investigated mechanisms underlying colour variation of immaculate, white avian eggshells. We specifically examined how the eggshell cuticle contributes to coloration. To do this, we experimentally removed the outer layers of immaculate, white eggshells of four species: chicken (Gallus gallus), Australian brush turkey (Alectura lathami), king pigeon (Columba livia domestica), and budgerigar (Melopsittacus undulatus). If the...
cuticle contributes to eggshell coloration, we predicted that its removal would cause a larger colour change in eggshells with cuticles compared to those without. We then used scanning electron microscopy, X-ray photoelectron spectroscopy, and chemical extractions to investigate if nanostructural features or chemical composition explain the observed patterns of coloration and its change following experimental manipulation.

RESULTS

Ultra High Performance Liquid Chromatography (UHPLC) and Mass Spectrophotometry (MS) confirmed that none of the eggshells of the four species (chicken, brushturkey, pigeon, and budgerigar) contained any detectable concentrations of protoporphyrin or biliverdin, whereas these pigments were detected in our positive controls (supplementary material Fig. S1).

Untreated eggs of the four species differed in overall structure, thickness and presence of cuticle (Fig. 1; Table 1). Chicken eggs were covered by a thin smooth cuticle that contained nanospheres with a mean diameter of 151.4±5.2 nm (n=40, s.e.m.). Brushturkey eggshells had a distinct cuticle composed of nanospheres with a mean diameter of 307.8±13.1 nm (n=40, s.e.m.). Pigeon eggshells had a smooth surface with some pores, and cross-section images for one of the eggs showed a structure resembling a very thin cuticle (supplementary material Fig. S2). Budgerigar eggshells lacked a cuticle, and the vesicles of the organic matrix were visible on the surface as pores with a diameter varying between 1–2 μm in diameter (Fig. 1).

Sequential treatment with ethylenediaminetetraacetic acid (EDTA) gradually removed the outer layers of all four species’ eggshells, but had differential effects on their structure (Fig. 1; supplementary material Fig. S3) and decrease in thickness (Table 1). After 30 min of EDTA treatment, the nanospheres of chicken eggshell cuticle were removed (supplementary material Fig. S3), whereas after 90 min of EDTA treatment, the cuticle was fully removed along with a portion of the underlying palisade layer (Fig. 1). After 30 min of EDTA treatment, only a few nanospheres were still present on the brushturkey eggshell (supplementary material Fig. S3), and after 90 min of EDTA treatment, parts of the underlying palisade layer became visible and removal of the cuticle was confirmed in the cross-section image (Fig. 1). After sequential EDTA treatment, the vesicles of the pigeon eggshell became gradually more distinct as deeper pores according to the time of the

Fig. 1. SEM images showing the different eggshell morphologies for untreated and EDTA treated eggs. The EDTA treatment durations are 90 min for chicken, brushturkey, pigeon, and 30 min for budgerigar. First and third column are cross-sections, second and fourth column are topview images. C=Cuticle layer. Scale bars are 10 μm.
treatment (Fig. 1; supplementary material Fig. S3). After 30 min of EDTA treatment, the holes on the budgerigar eggshell were still visible, however, the surface became much rougher and pockmarked (Fig. 1).

Gradual removal of the outer layers (including the cuticle if present) resulted in a significant increase in UV-chroma for chicken and brushturkey eggs. With increasing chemical etching of the outer layers, UV-chroma increased for chicken ($F_{1,11}=103.7, P<0.001$), brushturkey ($F_{1,17}=62.0, P<0.001$), and pigeon ($F_{1,8}=11.6, P<0.01$), but not for budgerigar ($F_{1,8}=1.8, P=0.22$) (Figs 2, 3; Table 2).

X-ray photoelectron spectroscopy (XPS) revealed the presence of phosphorus on the surface of chicken and brushturkey eggs, which completely disappeared following 90 min of EDTA treatment (Fig. 4; Table 3).

**DISCUSSION**

Despite the absence of known eggshell pigments (biliverdin and protoporphyrin), we found differences in the UV-reflectance of the four species’ eggshells. We showed that removal of the outer layers of avian eggshells that contain a cuticle increases UV-chroma, suggesting that the cuticle modulates UV-reflectance of white eggshells. This is likely achieved by selective absorption of UV-wavelengths by the compounds in the cuticle. The effects of the cuticle on eggshell coloration are particularly important, because the composition, thickness and extent of coverage of the cuticle (and thus potentially colour of the shell) can vary according to female age and egg freshness (Rodríguez-Navarro et al., 2013). These results highlight the importance of factors other than biliverdin and protoporphyrin in influencing avian eggshell coloration.

Eggshell colour varied across these unpigmented eggshells, and differed from that of pure calcite, even after their cuticles were removed (supplementary material Fig. S4). Although avian eggshells consist of approximately 96% calcite overall (Hamilton, 1986), the underlying structure of calcite crystals, or the composition of the organic matrix, can differ among species (Panheleux et al., 1999). These differences may cause variation in UV-chroma among the different species’ eggs studied here and highlight a role of non-pigmentary chemical or structural differences in influencing avian eggshell coloration. The chicken eggshell is particularly interesting as its UV-chroma drastically increased following removal of its outer layers. This finding suggests that some characteristic of the chicken eggshell increases the inherent UV-reflectance of calcite (supplementary material Fig. S4), possibly through nanostructuring as no identified pigment absorbs light across all wavelengths except UV (Andersson, 1999); however, the exact mechanism requires further investigation.

The increase in UV-chroma associated with removal of the outer eggshell layers was highest for eggshells with a clearly defined
Table 2. The effects of sequential EDTA treatment on UV-chroma
(\textit{mean±s.e.m., n}=3).

<table>
<thead>
<tr>
<th>EDTA treatment (min)</th>
<th>Chicken</th>
<th>Brushturkey</th>
<th>Pigeon</th>
<th>Budgerigar</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>0.12±0.11</td>
</tr>
<tr>
<td>20</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>0.01±0.11</td>
</tr>
<tr>
<td>30</td>
<td>1.40±0.95</td>
<td>1.69±1.13</td>
<td>0.16±0.39</td>
<td>0.15±0.28</td>
</tr>
<tr>
<td>60</td>
<td>2.30±0.86</td>
<td>1.27±1.06</td>
<td>0.32±0.40</td>
<td>n/a</td>
</tr>
<tr>
<td>90</td>
<td>3.73±1.46</td>
<td>1.87±0.58</td>
<td>0.66±0.16</td>
<td>n/a</td>
</tr>
<tr>
<td>120</td>
<td>4.46±1.59</td>
<td>2.58±0.33</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>150</td>
<td>n/a</td>
<td>2.97±0.74</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>180</td>
<td>n/a</td>
<td>3.56±0.65</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>
biliverdin positive control. Briefly, shell samples were broken into small fragments (surface area $\sim 1 \text{ cm}^2$ and/or weight $\sim 400 \text{ mg}$), rinsed with distilled water, 70% ethanol and homogenized by grinding; then 1 ml of aqueous solution of disodium ethylenediaminetetraacetic acid (EDTA) pH 7.2 (100 mg/ml) was added, and the tubes were vortex-mixed for 1 min and centrifuged at 15,000 $g$ for 30 s in an Eppendorf 5430R Centrifuge, discarding the supernatants. This procedure was repeated three times and then 1 ml of acetonitrile-acetic acid (4:1 v/v) was added. The tubes were vortex-mixed for 2 min in 30 s bursts (and opened to allow the escape of CO$_2$), and subsequently centrifuged for 2 min at 15,000 $g$. The supernatants were then transferred to clean tubes and stored at 4°C in the dark until further analysis within 24 h. An aliquot was measured in a NanoDrop 2000c spectrophotometer for its UV-Vis absorbance spectrum from 250–700 nm versus acetonitrile-acetic acid as a blank. Pigment presence or absence was indicated from these spectra and confirmed and quantified by Ultra High Performance Liquid Chromatography (UHPLC) and Mass Spectrophotometry (MS). All shell extracts (whether or not pigment was detected by methods above) were further analysed through MS ion detection at specific masses (563 m/z for protoporphyrin and 583 m/z for biliverdin) to detect presence of pigments below the detection threshold of standard MS analysis. All observed pigments were also compared to commercially obtained standards of the free acids of biliverdin and protoporphyrin from Frontier Scientific Inc. (UT, USA) dissolved in acetonitrile-acetic acid.

**Experimental removal of outer layers**

To experimentally investigate the contribution of the cuticle to the optical properties of the eggshells, we sequentially removed the outer eggshell layers (including the cuticle if present) over a course of treatments. For each treatment, we floated eggshells (with their surface down) on a weak alkaline solution (pH 8.1) of 0.37M EDTA and then gently brushed the surface using soft tissue paper (Baker and Balch, 1962; Igic et al., 2015). We repeated this over a course of treatment times depending on the thickness of the eggshells: successive increments of 10 min for budgerigar and increments of 30 min for chicken, brushturkey, and pigeon. We repeated treatments until the eggshells became too thin and fragile to handle (30 min for budgerigar, 90 min for pigeon, 120 min for chicken and 180 min for brushturkey). The removal of the outer layers was visualised by SEM after 30 and 90 min of EDTA treatment (or only after 30 min for the budgerigar).

**Scanning electron microscopy (SEM)**

We mounted untreated and EDTA-treated eggshell fragments onto aluminium stubs, allowing the visualisation of both the shell surface and cross-section, which we then sputter-coated with gold/palladium for 3 min. SEM (JSM7401F, JEOL Japan) images were taken at a working distance of 8 mm with an accelerating voltage of 5 kV.

**Spectrophotometry**

We measured diffuse reflectance on eggshell fragments between 300 and 700 nm. To minimize geometric variation associated with shell curvature and rough surfaces, we measured reflectance from the flattest part of fragments taken from the equatorial region of eggs. We used an integrating sphere (AvaSphere-50-REFL) with a black gloss trap to exclude specular reflectance, an AvaSpec-2048 spectrometer, and an AvaLight-XE pulsed xenon light source (Avantes Inc., Broomfield, CO, USA). All reflectance

**Table 3. Chemical composition (atom percentages, %) before and after EDTA treatment determined by XPS.** Values indicating ND (not detectable) are below detection limit. EDTA treatment was 90 min for chicken, brushturkey and pigeon, and 30 min for budgerigar.

<table>
<thead>
<tr>
<th></th>
<th>Chicken</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>untreated</td>
<td>EDTA</td>
<td>untreated</td>
<td>EDTA</td>
<td>untreated</td>
<td>EDTA</td>
<td>untreated</td>
</tr>
<tr>
<td>C</td>
<td>64.7</td>
<td>59.6</td>
<td>39.4</td>
<td>60.0</td>
<td>67.3</td>
<td>64.4</td>
<td>69.4</td>
</tr>
<tr>
<td>O</td>
<td>23.3</td>
<td>27.2</td>
<td>40.5</td>
<td>28.0</td>
<td>24.8</td>
<td>27.6</td>
<td>23.2</td>
</tr>
<tr>
<td>N</td>
<td>10.0</td>
<td>11.1</td>
<td>7.1</td>
<td>10.5</td>
<td>6.7</td>
<td>7.0</td>
<td>6.6</td>
</tr>
<tr>
<td>Ca</td>
<td>1.4</td>
<td>2.1</td>
<td>8.6</td>
<td>1.5</td>
<td>0.8</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>P</td>
<td>0.7</td>
<td>ND</td>
<td>4.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.5</td>
<td>0.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Fig. 4. XPS survey spectra showing the chemical composition of eggshells before and after EDTA treatment. The EDTA treatment duration are 90 min for chicken, brushturkey, pigeon, and 30 min for budgerigar. The sodium peak results from the residual presence of EDTA, and was not taken into account to calculate the atomic percentages.
measurements were taken relative to a diffuse white standard (WS-2, Avantes Inc.). We quantified UV-reflectance because this region showed the greatest level of variation for our samples. To evaluate changes in UV-reflectance, we calculated UV-chroma as a proportion of UV-reflectance from total reflectance ($R_{200-400}/R_{300-700}$) using the summary function of the R package PAVO (Maia et al., 2013). UV-chroma accounts for differences in total reflectance and thereby eliminates the confounding effect of eggshell thickness on our results. We then compared UV-chroma of eggshells across sequential EDTA treatments.

We used linear models to test if UV-chroma changed following sequential removal of the outer layers. For each species separately, we constructed models with UV-chroma as responses, egg ID as discrete predictor and EDTA treatment as continuous predictor. We constructed models using normal error distribution and identity link functions (supplementary material Table S1). We analysed each species separately because: (i) EDTA treatment durations were not quantitatively the same for the four species because of their differences in eggshell thickness and (ii) it was unclear whether EDTA treatment had the same effects for all other species’ eggs. P-values were adjusted following Holm’s method (Aickin and Gensler, 1996). All statistical tests were implemented in R v.3.0.1 (R Development Core Team, 2013).

X-ray photoelectron spectroscopy (XPS)
The survey spectra of untreated and EDTA-treated eggshells (90 min for chicken, brush turkey, and pigeon eggs; and 30 min for budgerigar eggs) were collected using a VersaProbe II Scanning XPS Microprobe from Physical Electronics (PHI), under ultrahigh vacuum conditions with a pressure of $2 \times 10^{-6}$ Pa. Automated dual beam charge neutralization was used during the analysis of the samples to provide accurate data. The analyser pass energy was 117.4 eV and each spectrum was collected using a monochromatic Al Kα X-rays (hv=1486 eV) over a 200 μm diameter analysis area. The survey scans were used to evaluate the near surface region elemental composition of the eggshells. Peak areas were measured for the C 1s, O 1s, Ca 2p, N 1s, P 2p and S 2p regions and elements were quantified using instrument-modified Schofield cross sections (PHI MultiPak software). The sodium peak results were calculated UV-chroma as a proportion of UV-reflectance from total reflectance.

Acknowledgements

Author contributions

Author correlations of the manuscript. D.F.L. and B.I. developed the experimental approach. D.F.L. performed SEM, spectrometry and XPS. A.V. and M.H. performed the pigment extraction. M.E.H. and L.D. provided the biological samples. M.S. directed the project.

Funding

This research was funded by the Human Frontier Science Program (RGY83/2012) to MEH, TG, MDS, and GINW. MH and AV acknowledge funding support from National Science Foundation (NSF) award 1247550 and the Camille and Henry Dreyfus Foundation. MDS acknowledges support from AFOSR grant 9550-13-1-0222. DH and TG were funded by the European Social Fund and the state budget of the Czech Republic, project no. CZ.1.07/2.3.00/30.0041.

References


Supplementary material Fig. S1. Mass spectra of chicken and brushturkey eggshell extracts are shown as example of eggs that lack a detectable amount of protoporphyrin (upper three) and biliverdin (lower three).
**Supplementary material Fig. S2.** Cross-sectional SEM image of one particular pigeon egg showing a structure resembling a very thin cuticle (C). Scale bar is 10 µm.

**Supplementary material Fig. S3.** Effect of 30 min EDTA treatment on the surface morphologies of chicken, brushturkey and pigeon.
Supplementary material Fig. S4. Diffuse reflectance of a thin, flat layer of pure calcite powder (Sigma Aldrich, St. Louis, MO, USA), compared to those of untreated (solid lines) and EDTA-treated (dashed lines) eggshells.
Table S1. Summary output for linear models comparing the change in UV chroma in relation to EDTA treatment.

<table>
<thead>
<tr>
<th>Species</th>
<th>UV chroma(^i)</th>
<th>95 % C.I.</th>
<th>Term(^ii)</th>
<th>F</th>
<th>dfs</th>
<th>(P^{iii})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>3.92e-02 ± 0.38e-02</td>
<td>[3.07e-04, 4.77e-04]</td>
<td>Egg ID</td>
<td>3.33</td>
<td>2, 11</td>
<td>0.074</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EDTA</td>
<td>103.66</td>
<td>1, 11</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Brushturkey</td>
<td>1.73e-02 ± 0.22e-02</td>
<td>[1.27e-04, 2.19e-04]</td>
<td>Egg ID</td>
<td>5.93</td>
<td>2, 17</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EDTA</td>
<td>61.95</td>
<td>1, 17</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Pigeon</td>
<td>0.71e-02 ± 0.21-02</td>
<td>[0.23e-04, 1.20e-04]</td>
<td>Egg ID</td>
<td>5.43</td>
<td>2, 8</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EDTA</td>
<td>11.62</td>
<td>1, 8</td>
<td>0.009</td>
</tr>
<tr>
<td>Budgerigar</td>
<td>0.62e-02 ± 0.47-02</td>
<td>[-0.45e-04, 1.70e-04]</td>
<td>Egg ID</td>
<td>1.09</td>
<td>2, 8</td>
<td>0.380</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EDTA</td>
<td>1.78</td>
<td>1, 8</td>
<td>0.219</td>
</tr>
</tbody>
</table>

\(^i\)Change in UV chroma (%) per min of EDTA treatment ± SE  
\(^ii\)Egg ID: number of egg; EDTA: time of EDTA treatment  
\(^iii\)P-values for EDTA were adjusted following Holm’s method