

First light for avian embryos: eggshell thickness and pigmentation mediate variation in development and UV exposure in wild bird eggs

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Summary

1. The avian embryo's development is influenced by both the amount and the wavelength of the light that passes through the eggshell. Commercial poultry breeders use light of specific wavelengths to accelerate embryonic growth, yet the effects of the variably patterned eggshells of wild bird species on light transmission and embryonic development remain largely unexplored.
2. Here, we provide the first comparative phylogenetic analysis of light transmission, through a diverse range of bird eggshells (74 British breeding species), in relation to the eggshell's thickness, permeability, pigment concentration and surface reflectance spectrum (colour).
3. The percentage of light transmitted through the eggshell was measured in the spectral range 250–700 nm. Our quantitative analyses confirm anecdotal reports that eggshells filter the light of the externally coloured shell. Specifically, we detected a positive relationship between surface eggshell reflectance ('brightness') and the percentage of light transmitted through the eggshell, and this relationship was strongest at wavelengths in the human-visible blue-green region of the spectra (*c.* 435 nm).
4. We show that less light passes through thicker eggshells with greater total pigment concentrations. By contrast, permeability (measured as water vapour conductance) did not covary significantly with light transmission. Eggs of closed-nesting species let more light pass through, compared with open nesters.
5. We postulate that greater light transmission is required to assist embryonic development under low light exposure. Importantly, this result provides an ecological explanation for the repeated evolution of immaculate, white- or pale-coloured eggshells in species nesting in enclosed spaces.
6. Finally, we detected correlative support for the solar radiation hypothesis, in that eggshells of bird species with a longer incubation period let significantly less of the potentially harmful, ultraviolet (UV) light pass through the eggshell. In summary, we demonstrate suites of avian eggshell properties, including eggshell structure and pigmentation, which are consistent with an evolutionary pressure to both enhance and protect embryonic development.

Key-words: breeding birds, eggshell colour, light transmission, nesting behaviour, pigments, ultraviolet light

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Introduction

The avian eggshell is a complex multifunctional bioceramic (Fernandez, Araya & Arias 1997). It actively shapes the developmental milieu of the avian embryo by protecting it from mechanical damage (Birchard & Deeming 2009), facilitating gas exchange (Ar *et al.* 1974; Ar & Rahn 1985; Portugal *et al.* 2014), and providing calcium for bone growth (Richards & Packard 1996). Amongst wild birds, both the structure and appearance of eggshells are finely tuned to a particular incubation microenvironment (Bakken *et al.* 1978; Cassey *et al.* 2010). Whereas the eggshell's role in protecting the embryo from the physical elements is obvious, it is less clear to what extent the shell mediates different environmental factors reaching the embryo, to either enhance or diminish their effects. This is particularly true for the transmission and filtering of external light (Maurer, Portugal & Cassey 2011). Exposure to ultraviolet (UV-B) radiation, for example, can have detrimental effects on the developing embryo through the production of DNA lesions (Tevini 1993; Thoma 1999). Conversely, UV-B light exposure is essential for calcium metabolism (Stanford 2006), and exposure may also benefit the avian embryo by killing harmful micro-organisms on the eggshell surface (Scott 1993) and, potentially, through the interaction with photo-active eggshell pigments possessing antimicrobial properties (Ishikawa *et al.* 2010). Eggshell pigments are also likely to play a key role in mediating the interactions between external light and the avian embryo, as they can act as a general barrier (Cooper *et al.* 2011) or as a wavelength-specific filter to generate a particular light environment (Lahti 2008). These functions may provide hitherto unrecognized adaptive roles or evolutionary constraints to the diversity of eggshell coloration and appearance.

A recent review of eggshell pigmentation (Maurer, Portugal & Cassey 2011) summarized the evidence for the beneficial effects of certain light environments (defined as specific wavelengths and temporal patterns of light exposure) on the development of avian embryos. In particular, the specific wavelength of the light, which an egg is exposed to, can influence both the speed of embryonic development (Lauber 1975; Shafey & Al-mohsen 2002) and the behavioural performance of chicks (Rogers & Krebs 1996). Studies on domesticated avian species have shown that short periods of white or green light (*c.* 560 nm) exposure can reduce incubation time by almost one day (Ghatpande, Ghatpande & Khan 1995; Rozenboim *et al.* 2004). Blue light (*c.* 500 nm) is particularly effective and beneficial in establishing a circadian rhythm (Csernus, Becher & Mess 1999) that bird embryos, unlike mammal embryos, cannot directly establish via hormones in the maternal blood stream, but can do so indirectly from the modulation of incubation rhythms by the parent birds (Zeman *et al.* 1999; Cooper *et al.* 2011). While the exact sensory, chemical, and physiological mechanisms behind these beneficial effects have not yet been determined, the evolution of photolyase repair of UV-induced

DNA dimers in birds, but not mammals (Van de Merwe & Bronk 1981; Hearst 1995), suggests that the interactions with the light environment are an integral component of avian embryonic development. It remains unclear, however, what role (if any) eggshell features, including structure and pigmentation, play in modulating the light environment of the embryo in nature (Maurer, Portugal & Cassey 2011).

For wild birds, Max Schönwetter (Schönwetter 1960–1992), the leading oologist of his time (Maurer, Russell & Cassey 2010), first explored the human-perceived correlation between eggshell colour and the light environment inside the shell. In his visual assessments, Schönwetter noted that (to the human eye) the light visible on the inside of the egg (observed through the blowhole) frequently is of a similar hue as the outside reflected colour. This suggests that the entire eggshell, rather than the pores alone, is permeable to light and hints at a possible complex role of the eggshell as a wavelength-specific light filter. If confirmed, this could provide a chemical–physical basis for selection on structural eggshell traits, such as pigmentation and thickness, affecting the rate and course of embryonic development (Maurer, Portugal & Cassey 2011). In particular, we predict that the different positive effects of light transmission, already demonstrated for domestic poultry (Shafey & Al-mohsen 2002; Shafey 2004), may also have considerable adaptive values for wild bird's eggs and development. For example, artificial light transmitted through the eggshells of domestic poultry accelerates embryonic development (Ghatpande, Ghatpande & Khan 1995; Rozenboim *et al.* 2004; Shafey 2004). If this acceleration also occurs in wild-nesting birds, in response to natural light exposure (e.g. seasonal or latitudinal) or parentally mediated light exposure (e.g. nest site choice, nest construction, incubation rhythm), it could reduce the species' incubation period and thus the breeding birds' risk of losing the clutch to predation or inclement weather.

In this study, we measured the transmission of light (% wavelength) through the avian eggshell in relation to shell thickness and permeability (measured as water vapour conductance; G_{H_2O}) for the eggs of 74 British and European breeding bird species (both passerines and non-passerines). We also assessed the extent and direction of the correlation between the wavelength of the internal light transmitted through the shell and the external light reflected from the surface of the shell. The resulting data were used to evaluate the potential role of shell appearance and pigment concentration in modulating avian embryonic developmental environments. Specifically, we predicted the following patterns of physical properties of light transmission through the eggshell: (i) species with thicker eggshells let less light pass through, whereas (ii) species with greater water vapour conductance through the eggshell, and thus potentially wider or more numerous eggshell pores (Ar *et al.* 1974), allow more light to be transmitted, and (iii) species with greater eggshell pigment concentrations allow less light to transmit through the shell.

In addition to the physical properties of eggshells, we also assessed whether light transmission patterns across the eggs of wild bird species are consistent with a protective and developmental function of eggshell pigmentation. Here, we predicted that (iv) the eggshells of species nesting in 'closed' environments (e.g. in holes, cavities, and burrows) would let more light pass than those of open nesters, facilitating the beneficial developmental effects of additional light and daily patterns of light–dark cycles (Cooper *et al.* 2011). Testing this hypothesis may contribute to a novel explanation for the long-established pattern of the repeated and consistent evolution of white or light-blue eggshells in cavity-nesting birds (Kilner 2006). Finally, we predicted that (v) species with longer incubation periods, and thus presumably greater exposure to harmful UV light (Maurer, Portugal & Cassey 2011; Beckmann *et al.* 2014), would have eggshells that permit less UV-wavelength light to pass than birds with shorter incubation stages. We expected that this would be more prevalent amongst open-nesting species. Such a finding would lend empirical (but indirect) correlative support to the solar radiation hypothesis, which predicts that eggshell pigmentation reduces exposure of the embryo to harmful UV light (Lahti 2008).

Materials and methods

SAMPLE SELECTION AND PREPARATION

We selected eggshell specimens of British and European breeding bird species held in the 'Class II data poor' egg collection at the Natural History Museum (NHM) in Tring (Portugal, Maurer & Cassey 2010; Russell *et al.* 2010). We sampled three eggs of one species from every genus (Sibley & Monroe 1990) represented in the collection with three or more specimens. Each eggshell specimen was cut in half longitudinally and weighed to an accuracy of 0.001 g on a Mettler PC 440 digital scale. Only eggs that had no visible cracks, were free of dirt inside and outside, and possessed an intact inner membrane were used. For genera with both open-nesting and closed-nesting species, we sampled eggs from one open-nesting and one closed-nesting species. Our choice of samples was constrained by the parameters of the light transmission spectrophotometer. Preliminary observations using the eggs of 230 European bird species (Maurer, Portugal & Cassey 2012) showed that the spectrophotometer light source, for measuring percentage light transmitted (see details below), was not strong enough to allow any detectable light transmission for eggshells with a thickness of greater than 0.45 mm (such as Common Guillemots *Uria aalge*). Eggs of domestic chickens have thicknesses just below this threshold (Shafey 2004). Similarly, eggs with a length less than 25 mm and a thickness less than 0.1 mm proved too fragile and did not withstand sampling and were excluded from analysis. The smallest and the largest egg we sampled, respectively, were that of the Eurasian Hoopoe *Upupa epops* and the Common Eider *Somateria mollissima*. The complete list of the 74 species used in our study (70 genera in 23 families; 20 closed-nesting species) is provided as Supplementary Material.

We measured spectrophotometric reflectance, water vapour conductance (through the shell) and eggshell thickness (at the equator) on the same individual eggshell samples. One half was used for calculating light transmission, while the other half was used for estimating water vapour conductance. We quantitatively determined the concentration of the two known avian eggshell

pigments biliverdin and protoporphyrin IX (Gorchein, Lim & Cassey 2009) for a subset of these species ($n = 31$) using eggshell samples from the same source of the NHM Class II collection, where available (See Methods below).

LIFE HISTORY DATA

Data on nest type and incubation length (days), for each species, were obtained from the Birds of the Western Palaearctic (Cramp & Simmons 1978–1994). Nest type was scored as either 'open' (0) or 'closed' (1). A species' nest was classified as closed if it was recorded as being commonly placed in a ground cavity, excavated burrow or under the cover of boulders or in a covered nest, crevice, tree hollow or artificial nest box.

LIGHT TRANSMISSION MEASUREMENTS

We used a UV-2401 PC Shimadzu (Kyoto, Japan) UV-VIS recording spectrophotometer for all light transmission measurements. Eggshells were cut longitudinally into roughly rectangular segments c. 10 mm in width and >15 mm upwards and downwards from the equator in height, to fit upright into specifically constructed open-sided plastic cuvettes. The cuvettes allowed the light to reach the eggshell directly and unhindered. The shells were fixed in the spectrophotometer so that the outer surface was facing towards the light source. A blind with an opening 8 mm wide and 25 mm high was placed in the light beam, in front of the eggshell, to ensure that no light would pass around the eggshell segments and interfere with the measurement. Percentage transmission, measured through eggshell segments with this blind in place, was of the same order of magnitude as that found by Shafey *et al.* (2004) for chicken eggs.

Prior to each series of measurements, the UV-2401 was run for c. 5 min to allow the lamp to warm-up, ensuring consistent readings. The spectrophotometer was calibrated, for light transmission without any material in the light beam and with the blind in place, as follows: The recording range was set to 'Low 0' to 'High 2', and the wavelength range started at 800 nm and ended at 200 nm with a medium scan speed, a slit width of 5.0 mm, and a sampling interval of 2 nm as presented in Shafey *et al.* (2004).

At 380 nm the light filter changed (automatically) to allow for the dual detection of UV and longer wavelength spectra. We excluded the region between 360–410 nm from analyses to avoid any spurious effects associated with the change in filter (B. Jackson pers. comm.). Light transmission was recorded as the percentage (%) compared to a blank ('empty') sample (i.e. no obstruction in the light path = 100% transmission).

SHELL THICKNESS

Eggshell thickness indices have been previously quantified, and repeatability was reported by us for 230 European species (Maurer, Portugal & Cassey 2012). Following the same procedure, shell thickness was measured in the 74 species using a specifically modified constant measurement force micrometre (Mitutoyo Series 227–203) to an accuracy of 1 µm using a measurement force of 1.5N as previously described (Maurer, Portugal & Cassey 2011). Three measurements were obtained at the equator for each sample (after the eggshell was cut in half), and the mean was calculated for further analysis. In our species ($n = 74$), eggshell thickness (mm) was positively, and significantly, correlated with other measures of physical eggshell size (e.g. eggshell length; mm: Pearson's r [95% CI] = 0.875 [0.808, 0.920], $t = 15.35$, $P < 0.001$). Given the intuitive hypothetical relationship between light transmission through the avian shell and eggshell thickness, we used eggshell thickness in all subsequent analyses as our measure of eggshell size.

WATER VAPOUR CONDUCTANCE

Water vapour conductance through the eggshell was determined using the methodology described in detail, and validated, by Portugal, Maurer & Cassey (2010) and is a previously determined measure of eggshell permeability (Ar *et al.* 1974). Briefly, we glued intact fragments of the eggshells on top of 250- μL microtest tubes filled with 200 μL of distilled water. The tubes were placed in a desiccator at a constant temperature of 25 °C and weighed (in grams to four decimal places) three times at 24-h intervals. Any mass loss was assumed to be the result of water evaporation through the pores and was expressed as water loss per 24 h corrected to standard barometric pressure ($\text{mg day}^{-1} \text{ torr}^{-1}$) (Portugal, Maurer & Cassey 2010).

EGGSHELL PIGMENT CONCENTRATIONS

Where possible, eggshell pigment concentrations were obtained from the same eggshell samples used in the light transmission analysis, as well as others. In total, 31 species (average 6 samples per species; min = 4, max = 13, from 13 families including 7 cavity nester species) were available for pigment analysis. Both types of the known avian eggshell pigments, protoporphyrin IX and biliverdin (Gorchein, Lim & Cassey 2009) were quantified as their dimethyl esters following Mikšík, Holan & Deyl (1996) and as described in detail in Cassey *et al.* (2012c). The concentration of the two pigments is positively correlated within these species (Cassey *et al.* 2012c), and it is not straightforward to predict eggshell colour from the varying concentrations of the individual pigments (Cassey *et al.* 2012a,b,c). Given that we are interested in the proposed influence of total pigment concentration hindering the transmission of light through the eggshell, we summed the total pigment concentrations (\log_{10} -transformed) for all subsequent analysis.

REFLECTANCE SPECTROPHOTOMETRY

We measured the reflectance between 250 and 700 nm using an Ocean Optics USB2000 Miniature Fibre Optic Spectrophotometer with illumination by a DT mini-lamp. The identical samples used for the light transmission analysis were used for these measurements. Following the manufacturer's instructions, the lamp was run for 15 min before the measurements to ensure consistent light production. The probe was fitted with a custom-built light-proof cap, which was fixed at a consistent measurement angle of 90° (Cassey *et al.* 2012a). Spectra were expressed relative to a white Ocean Optics WS-1 diffuse reflectance standard and measured in 0.4-nm steps. Three representative (systematic) spectrophotometric recordings were taken on each fragment (Fig. S1), at c. 0.25, 0.50 and 0.75 of the sample height, to capture the latitudinal variation in eggshell appearance. Measurements were conducted by a single observer (GM) and were taken without specifically choosing

background colour or maculation as the point of measurement. This approach was followed since the light transmission measurements could, in practice, only be taken across the shell integrating both background and maculation. All spectra were visually assessed for wavelength aberrations (e.g. stray light entering the probe) before cataloguing. Regular calibration (c. every 15 min) with dark and white standards ensured that instrument error or shift was kept to a minimum.

STATISTICAL ANALYSES

All statistical analyses were conducted in the R software environment for statistical computing and graphics (R Core Team 2013). Average light transmission and eggshell reflectance were calculated in 5-nm steps using an interpolated average (Cassey *et al.* 2010). Transmission and reflectance measurements (%) are presented as the sum of the interpolated averages divided by the total length of the wavelength; analogous to 'brightness' (Montgomerie 2006). Light transmission percentages were log-transformed (\ln) for analysis. Repeatability of a species' light transmission between replicate eggshells was quantified using the intra-class correlation coefficient, ICC (Nakagawa & Schielzeth 2010) in the R package *ICC* (Wolak, Fairbairn & Paulsen 2012). Light transmission and reflectance were calculated across the range 250–700 nm. Transmission for UV and visible light was analysed separately where appropriate. UV transmission and reflectance were calculated between 250 and 360 nm (Shafey *et al.* 2004), and the transmission/reflectance of longer wavelength (i.e. human-visible) light was calculated between 410 and 700 nm to account for the range excluded from the transmission measurements, and including both blue-green (410–575 nm) and red (580–700 nm) chromatic regions of the wavelength. The blue-green appearance of many birds' eggshells has always been of particular evolutionary interest to ornithologists (Lack 1958; Kilner 2006).

Phylogenetic generalized least square (PGLS) models were constructed using the *pgls* function in the package *caper* (Orme *et al.* 2011). The *pgls* function incorporates the co-variance between taxa into the calculation of estimated coefficients from a generalized least squares model. The co-variance matrix, of the expected co-variance between each pair of tips, is calculated using the branch lengths of an estimated phylogeny. We used the genetic sequence-based phylogeny of British birds constructed by Thomas (2008) and updated by (Cassey *et al.* 2012c).

We tested the predictions (i–vi) of each of the hypotheses described above (see Introduction) by comparing the performance of models with multiple terms to a global model with all of the univariate terms included. The statistical significance of the variables was assessed as linear terms in the PGLS models ($\alpha = 0.05$). Model performance was assessed by changes in the model's Akaike Information Criterion corrected for small sample sizes (AICc). Model estimates (with standard errors) and relative model weights are presented for these analyses (Table 1). We also tested for second-order interactions between nest type (open versus

Table 1. Model support (Akaike's information criterion; AICc) for the top-ranked PGLS models ($\Delta\text{AIC}_C < 6.0$) of light transmission through the eggshell ($n = 74$ species). Predictor variables (estimates [and standard errors]) are provided for the terms retained in each model. Relative model weights were estimated across the entire global model set, and sum to one

Intercept	Thickness (mm)	Conductance ($\text{mg day}^{-1} \text{ torr}^{-1}$)	Nest-type (Closed)	Incubation (days)	AICc	ΔAIC_C	Relative model weight
−3.78 [0.64]	−3.34 [1.03]		0.77 [0.24]		199.8	0.00	0.423
−4.08 [0.66]	−3.74 [1.74]	1.71 [1.06]	0.81 [0.25]		201.3	1.48	0.203
−3.97 [0.71]	−3.99 [2.11]		0.71 [0.26]	−0.01 [0.02]	201.6	1.77	0.175
−4.18 [0.71]	−4.11 [2.10]	1.63 [1.07]	0.77 [0.26]	−0.01 [0.02]	202.9	3.11	0.089
−4.40 [0.70]			0.76 [0.26]	−0.01 [0.02]	204.0	4.24	0.051

closed) and all other variables. We calculated Pagel's Lambda, estimated by maximum likelihood, to assess the strength of phylogenetic signal in the models. Lambda varies from 0 to 1 where 0 indicates no phylogenetic signal in the data and 1 is consistent with a Brownian motion model of trait evolution, in which the phylogeny accurately reflects the co-variances between species for a given trait (Freckleton, Harvey & Pagel 2002).

Results

LIGHT TRANSMISSION THROUGH THE EGGSHELL

There was considerable variability in the average percentage of light transmitted through the shell across the different species (see Results below). On average, the percentage of light transmitted through the eggshell, at a given wavelength, was very small (<0.1%; Fig. 1). Within species (across replicate shell samples), average light transmitted through the shell was highly repeatable (ICC [95% CI] = 0.912 [0.874, 0.941]) and comparable (for example) with the repeatability of average eggshell sample thickness measured at the shell equator (ICC [95% CI] = 0.940 [0.916, 0.959]).

Overall, median species light transmission increased with increasing wavelengths between 250 and 700 nm (Fig. 1), and there was a positive relationship between eggshell reflectance and light transmission through the shell (Fig. 2). The positive relationship between eggshell reflectance and light transmission was strongest at 435 nm; this is in the human-visible blue-green region of the spectrum (Fig. S2).

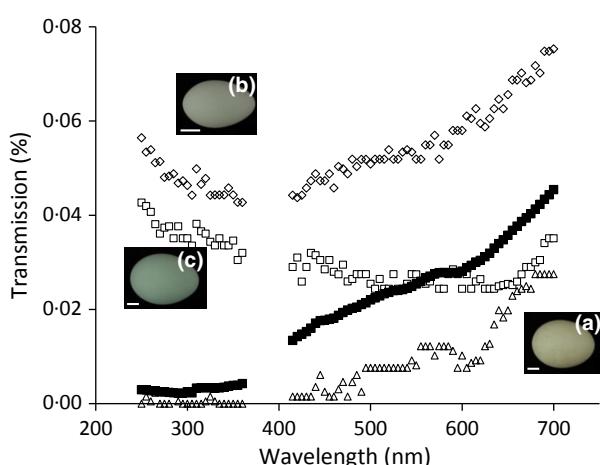


Fig. 1. The percentage of light transmitted through the eggshell for wavelengths at 5-nm intervals between 250 and 700 nm. Different symbols depict the median across 74 species (solid squares) and three individual eggshells (pictured; scale bar = 10 mm) for the species at the 25th-percentile of light transmission ((a) *Nyctea scandiaca*; hollow triangle) and 95th-percentile of light transmission ((b) *Tachybaptus ruficollis*; hollow diamond), and (c) *Ardea cinerea* (hollow square), the only species for which the average light transmitted through the shell in the UV wavelengths was greater than in the longer wavelength region of the spectra (see also Fig. S3).

PHYLOGENETIC MODELS OF EGGSHELL LIGHT TRANSMISSION

The phylogenetic signal in light transmitted through the shell was extremely strong. The maximum likelihood estimate of Pagel's Lambda ranged from 0.933 to 0.984 in the five top PGLS models for explaining eggshell light transmission (Table 1).

The best-supported phylogenetic model, for explaining the variability in light transmission through the eggshell, included eggshell thickness (mm) and nest type (open versus closed nesting) (Table 1). Thicker eggshells (mm) were associated with a significant reduction in the percentage of light transmitted through the eggshell (Fig. 3a), whereas the eggshells of 20 closed-nesting species permitted the transmission of a significantly greater percentage of light than open-nesting species (Fig. 3b). Interactions between nest type (closed versus open) and all other variables were not significant and did not contribute to any of the best-fitting models (Table 1). Water vapour conductance values ($\text{mg day}^{-1} \text{ torr}^{-1}$) for the eggshell, as a measure of eggshell permeability, had no significant effect on the amount of light transmitted through the eggshell (univariate PGLS estimate $\pm \text{SE} = 0.989 \pm 1.084$, $t_{73} = 0.913$, $P = 0.364$). Similarly, incubation length (days) had no significant effect on the amount of light transmitted through the eggshell (univariate PGLS estimate $\pm \text{SE} = 0.014 \pm 0.021$, $t_{73} = 0.641$, $P = 0.523$).

For a subset of samples with measured pigment concentrations ($n = 31$), eggshells of greater total pigment concentration ($\log_{10}[\text{nmol g}^{-1}]$) transmitted less light through the eggshell (Fig. 4; estimate $\pm \text{SE} = -0.826 \pm 0.177$, $t_{27} = -4.649$, $P < 0.001$). The negative relationship between eggshell thickness and light transmission, and the difference in light transmission levels between open and closed-nesting species ($n = 8$ out of 31), remained significant and marginally non-significant, for this smaller data set (thickness estimate $\pm \text{S.E.} = -8.360 \pm 2.017$, $t_{27} = -4.145$, $P \leq 0.001$; closed-nesting estimate $\pm \text{S.E.} = 0.500 \pm 0.253$, $t_{27} = 1.970$, $P = 0.059$).

UV-SPECIFIC LIGHT TRANSMISSION THROUGH THE EGGSHELL

The average light transmitted through the eggshell in the UV wavelengths (250–360 nm) was positively correlated (across the 74 species) with the average light transmitted in the longer wavelength (410–700) region (Fig. S3; Pearson's r [95% CI] = 0.54 [0.35, 0.68], $t = 5.38$, $P < 0.001$).

In the PGLS model, which included nest type (closed versus open), length of incubation (days), and eggshell thickness (mm) as explanatory variables (Table S1), eggshells of species with longer incubation periods showed significantly reduced UV transmission through the shell (estimate $\pm \text{S.E.} = -0.078 \pm 0.033$, $t_{69} = -2.338$, $P = 0.022$). This result was marginally non-significant for the smaller data set that included pigment concentration as an

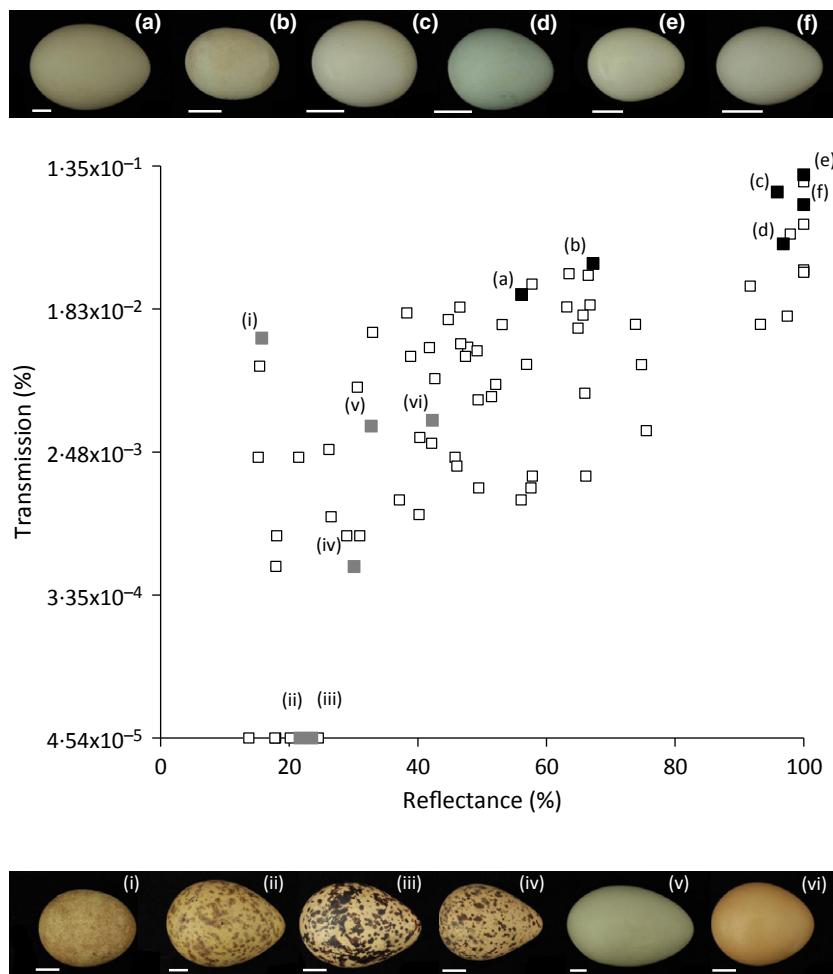


Fig. 2. Bivariate scatterplot of the positive relationship between the percentage of light reflected by the outside of the eggshell at 435 nm (in the blue-green region of the human-visible wavelength) and the percentage of light transmitted through the shell at that same wavelength for 74 species of breeding birds. As noted in the Results, the positive relationship between eggshell reflectance and light transmission was strongest at this region of the spectra (see Fig. S2). Highlighted points are associated with the species represented in the surrounding photos. Top panel (solid black points; in increasing order of percentage reflection [left to right]): (a) *Tadorna tadorna*; (b) *Hydrobates pelagicus*; (c) *Merops apiaster*; (d) *Sturnus vulgaris*; (e) *Picus viridis*; (f) *Cinclus cinclus*. Bottom panel (grey points; in increasing order of percentage reflection [left to right]): (i) *Falco subbuteo*; (ii) *Numenius arquata*; (iii) *Pluvialis apricaria*; (iv) *Tringa totanus*; (v) *Somateria mollissima*; (vi) *Phasianus colchicus*. The white scale bar in each photograph = 10 mm.

explanatory variable (incubation length estimate \pm S.E. = -0.178 ± 0.087 , $t_{27} = -2.046$, $P = 0.051$).

Contrary to our predictions, UV transmission was not significantly influenced by nest type (Table S1). Interactions between nest type (closed versus open) and all other variables (including incubation length) were not significant and did not contribute to any of the best-fitting models (all relative model weights $w_{i(AIC)} < 0.01$).

Discussion

The wavelength and intensity of light experienced by the avian embryo have the potential to affect its development in ways that could facilitate a species' adaptation to its specific nesting environment (Maurer, Portugal & Cassey 2011). It was previously proposed that the avian eggshell modulates characteristics of the light reaching the embryo

and could be capable of preventing light of harmful wavelengths from entering, while also facilitating the transmission of beneficial light (e.g. Lahti 2008). Here, we demonstrated that specific aspects of the eggshell's physical structure (thickness) and chemical structure (pigment concentration and shell reflectance) impact the light transmitted and thus experienced by the embryo, during development.

Similar to other bioceramics (e.g. dentine in human teeth; Hirmer *et al.* (2012)), we found that greater eggshell thickness reduced the amount of light transmitted through the eggshell for the range of species included in this study (Fig. 3a). However, it must be noted that our shell samples were derived from museum-sourced eggs, which were mostly collected during the early incubation stage (as indicated by small blowholes made for the preparation of the specimens; Maurer, Portugal & Cassey (2012)). Because

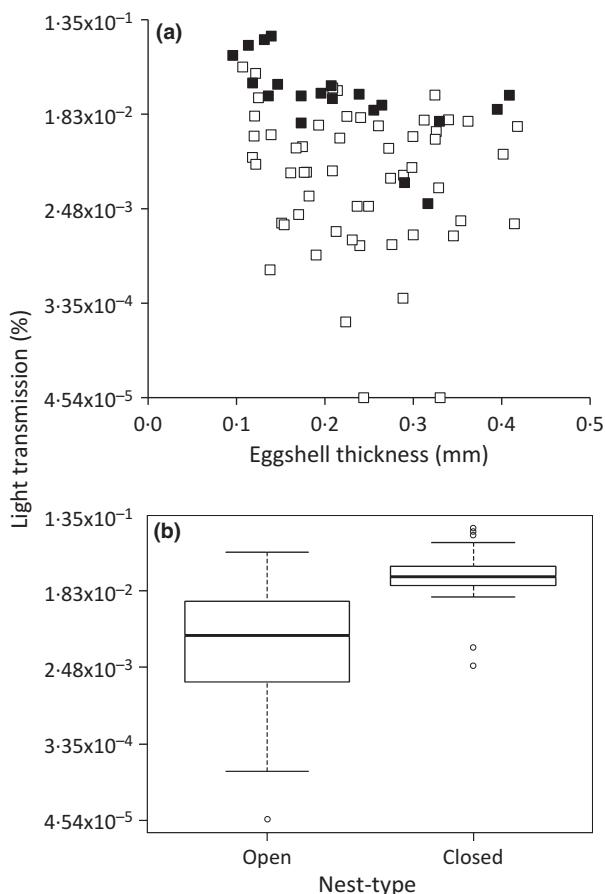


Fig. 3. (a) Bivariate scatterplot of the relationship between the light transmitted through the eggshell and eggshell thickness (mm; $n = 74$). Hollow points ($n = 54$) represent open-nesting species and solid points ($n = 20$) represent closed-nesting species. (b) On average, the eggshells of closed nesters ($n = 20$ species) have significantly greater light transmission through the shell than those eggshells of open nesters ($n = 54$ species). The two closed-nesting species with the lowest light transmission are auks (Aves: Alcidae) (*Cephus grylle* and *Fratercula arctica*). Despite appearing to be outliers, in both cases, these closed-nesting species had light transmission greater than any of their closest relative open-nesting species (*Stercorarius parasiticus*, *Stercorarius skua*, *Thalasseus sandvicensis*, *Chlidonias niger*, *Rissa tridactyla*), in their sister-clades Sternorariidae and Laridae.

eggshells are thin during development, as the embryo mobilizes the eggshell matrix to source calcium for its skeletal development (Richards & Packard 1996), our measurements, and estimates, may not be fully representative of the light transmission experienced by older embryos. Additional studies on the amount (and wavelength) of light transmitted through thinning eggs during incubation will provide a valuable parallel for our results generated here.

However, a second measure of the physical properties of the eggshell, shell permeability (measured as water vapour conductance), did not covary with light transmission through the shell. Pores may not facilitate light transmission as we predicted because their often complex structure and geometry (Mikhailov 1997) may act to diffuse and

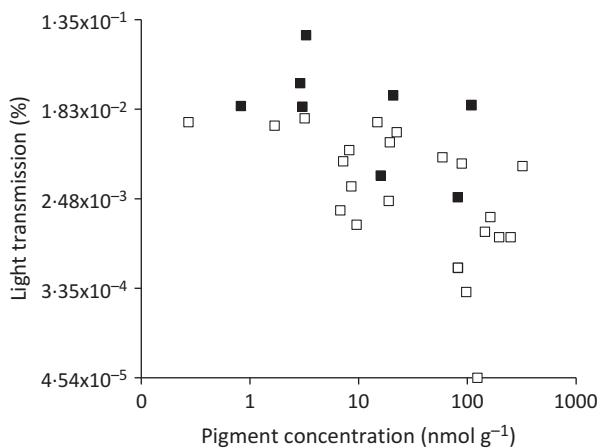


Fig. 4. Bivariate scatterplot of the relationship between the light transmitted through the eggshell and total pigment concentration (nmol g^{-1} ; $n = 31$). Hollow points ($n = 23$) represent open-nesting species and solid points ($n = 8$) represent closed-nesting species.

absorb the light waves rather than simply allowing passage through the shell. This will require direct measurements of pore structure, size and geometry to adequately quantify the impact of shell micro-structure on light transmission through avian eggshells.

In support of Schönwetter's observations, the transmitted light inside the eggshell corresponded to the colour reflected from the surface that is the pigmented colour 'shone through', and this was particularly evident in the blue-green region of the spectra (Fig. 2). Eggshell pigmentation, however, does not only operate as a colour filter. Indeed, for a subset of the species ($n = 31$), greater total concentration of eggshell pigments, both biliverdin and protoporphyrin together, reduced the percentage of light that passed through the shell (Fig. 4). The documentation of this pattern strengthens the evidence for the evolution of shell pigmentation as a light-blocking agent, at least in some wild species. In domestic chickens, pigment concentration also plays a crucial role in modulating light transmission, with greater pigment concentrations leading to reduced transmission (Shafey *et al.* 2004). The careful management of blue light has been particularly critical in this process as it is an effective stimulant of the embryonic circadian rhythm (Csensius, Becher & Mess 1999). Given the very strong association between the external shell reflectance and light transmission around the human-visible blue-green wavelength (435 nm), it remains to be shown, whether this function can help explain the long-standing questions regarding the evolution of blue-shelled pigmentation in wild birds (Lack 1958). We also consider that it will be particularly interesting to compare quantitatively the light-blocking functions in eggshell pigments with those exhibited by other bioceramic compounds (Comfort 1951).

As predicted, light transmission was higher in closed-nesting compared with open-nesting species, regardless of eggshell thickness (Fig. 3). This is consistent with an active

role of the shell in moderating the embryonic light environment. In addition, species with longer incubation periods, and thus greater light exposure, possessed eggshells with more effective UV blocking capacity, and this was independent of whether they were closed- or open-nesting species. From an avian embryo's perspective, it has been predicted that the optimal eggshell will inhibit harmful wavelengths while admitting beneficial light through the shell (Maurer, Portugal & Cassey 2011). Our comparative analysis of the eggs of 74 bird species provides results consistent with both functions. First, we showed that species with longer incubation periods, and thus greater exposure to light, have eggshells that permitted less UV light to pass. This may prevent UV light from harming embryonic development (Thoma 1999), but could also limit some of its positive effects (Stanford 2006). UV-B radiation is increasingly recognized as an important factor determining macro-ecological patterns (Beckmann *et al.* 2014), and the capacity of the eggshell to moderate UV light might prove important in explaining both the evolution of nesting habits and species distributions. Secondly, we provided evidence that the eggs of cavity-nesting species, which receive very little light (Cassey 2009; Holbeck *et al.* 2010), permitted more light, on average, to pass than eggs of open nesters. This may be necessary to assist with development (Shafey & Al-mohsen 2002; Shafey 2004) and to establish the embryonic circadian rhythm (Zeman & Gwinner 1993), in these extremely light poor environments. For example, in secondarily cavity-nesting House Sparrows *Passer domesticus*, greater light exposure speeds up embryonic development (Cooper *et al.* 2011).

A negative correlation between incubation period and light transmission is not unexpected given the role of thicker eggs in inhibiting the transmission of light through the eggshell. Larger, heavier species generally require both structurally stronger eggshells that support the weight of the incubating female without breaking (Birchard & Deeming 2009), and longer incubation periods to accommodate the development and growth of the larger embryo (Ricklefs 2010). However, the reduced light transmission in eggs experiencing longer incubation periods was not due to larger egg size or thicker shells alone. Instead, this association remained negative (and significant) when accounting for eggshell thickness, but was only statistically significant for UV wavelengths. It also persisted (marginally) when pigment concentration was accounted for. One explanation is that reduced light transmission may require prolonged incubation periods if light exposure speeds up embryonic development across taxa (as previously observed in House Sparrows: Cooper *et al.* 2011). In this, case we expect an unknown adaptive benefit of prolonged incubation to occur that outweighs the known costs such as increased predation risk. Alternatively, reduced light transmission, especially in the harmful UV spectrum, may be an adaptation by species with already longer incubation periods, to protect the embryo during prolonged development. Finally, because our data set is correlational, due to its

comparative origin, it is possible that the negative relationship between incubation period and light transmission is not linked physiologically or evolutionarily and may be explained by other, confounding but unmeasured ecological or physiological traits. These interesting findings remain to be tested in further experiments. Eggshell permeability, on the other hand, was found to have no effect on light transmission through the shell.

The role of eggshell maculation, including intricate spotting and scrawling (Hauber 2014) rather than homogenous pigmentation, in modifying the embryonic light environment was not explored in the present study as the equipment did not allow for the localized measurement of light transmission through areas of variable maculation. One aspect of maculation that is characteristic of many bird species is a concentration of the maculation (halo) that can be found at the blunt end of the egg (Schönwetter 1960–1992). This corresponds with where the air sac is located and where the head of the older embryo comes to lie during the later developmental stages (Burley & Vadehra 1989). The difference in light transmission between this area and other shell regions may aid the embryo in adjusting its orientation correctly and provide it with specific protection prior to hatching. The information content represented by the coloration and maculation of the blunt pole of the eggshell has also been found to critically influence egg rejection decisions by hosts of avian brood parasites (Poláčiková *et al.* 2011). Clearly, these fascinating scenarios of functional benefits (structural and signalling), costs, and their potential trade-offs warrant further investigation.

The avian eggshell influences the amount (percentage) and wavelength of light that reaches the avian embryo inside the egg. Levels of light transmission detected in this study were similar to those found in poultry eggs (Shafey *et al.* 2004); where light exposure has been shown to influence embryonic development. Importantly, we have identified a potential novel function of the eggshell in facilitating faster embryonic development in closed-nesting wild bird species. This function means that eggshell pigmentation may contribute to environmental adaptation in birds along gradients of light exposure such as greater levels of UV radiation at higher altitudes. As climate change forces birds to shift their distribution along such gradients (Bradshaw *et al.* 2014), we require specific information as to whether eggshell adaptations can keep track of a rapidly changing light environment. Future studies should focus on comparisons of eggs collected from different light environments, for example dense rain forests vs. open grasslands, and from different latitudes, to establish the generality of the pattern that we have demonstrated.

Conclusions

We have shown that variation in the embryonic light environment is predictable from the externally and easily obtained measures of eggshell size and pigmentation. We

further recommend comparative physiological and evolutionary research to assess eggshell traits (within clutches and across species) and determine how developmental parameters are affected by changing light environments in wild birds. Finally, we propose that our findings will provide a key comparison towards investigating, and answering, one of the most perplexing questions in the evolution of avian eggshell pigmentation; the prevalence of conspicuous blue eggs in small passerines (Lack 1958; Götmark 1992; Kilner 2006).

Acknowledgements

We thank Baz Jackson for his support and the access to the Transmission Spectrophotometer and Dan Hanley for comments on an earlier draft. Funding was provided by a Leverhulme Trust Research Project Grant, and in part by an HFSP Young Investigator Award. PC is an ARC Future Fellow (FT0991420). The authors have no conflict of interest.

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Received 10 November 2013; accepted 30 June 2014

Handling Editor: Barbara Tscharren

Supporting Information

Additional Supporting information may be found in the online version of this article:

Fig. S1. We measured the reflectance of external eggshell colours using an Ocean Optics USB2000 Miniature Fibre Optic Spectrophotometer with illumination by a DT mini-lamp. Three representative (systematic) measurements were taken on each fragment, at c. 0.25, 0.50, 0.75 of the sample height, to capture the latitudinal variation in eggshell appearance. These three measurements (grey lines) and their overall mean (black line) are provided for species of a range of background colours and maculation: (a) *Alectoris rufa*; (b) *Bucephala clangula*; (c) *Corvus monedula*; (d) *Crex crex*; (e) *Falco subbuteo*; (f) *Rissa tridactyla*; (g) *Tadorna tadorna*; (h) *Tringa totanus*. The white scale bar in each photo = 10 mm.

Fig. S2. Standardized effect sizes, from generalized linear mixed models at 5-nm intervals between 250 and 700 nm, for the positive relationship between eggshell reflectance and light transmission through the shell. Mixed models include the three replicate shell measurements from each species as a species-level random effect. The solid square indicates the specific relationship at the wavelength 435 nm (see Fig. 2).

Fig. S3. Bivariate scatterplot of the positive interspecific relationship between the average light transmitted through the eggshell, in the human-visible region of the spectra, and the average light transmitted in the ultraviolet (UV) wavelengths. The dotted line is the 1:1 relationship. Only one species (*Ardea cinerea*; see also Fig. 1) transmits more, on average, in the UV than in the longer wavelengths. Hollow points ($n = 54$) represent open-nesting species and solid points ($n = 20$) represent closed-nesting species.

Table S1. Model support (Akaike's information criterion; AICc) for the top-ranked pgl models of UV transmission through the eggshell ($n = 74$ species).

Appendix S1. Species list.