The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2012, **106**, 657–672. With 4 figures

Why are birds' eggs colourful? Eggshell pigments co-vary with life-history and nesting ecology among British breeding non-passerine birds

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Received 3 November 2011; revised 18 January 2012; accepted for publication 18 January 2012

The colourful appearance of bird eggshells has long fascinated biologists and considerable research effort has focused on the structure and biochemistry of the avian eggshell matrix. The presence of tetrapyrrole pigments was identified nearly a century ago. Surprisingly, how the concentrations of avian eggshell pigments vary among related species, and whether this variability is associated with either eggshell appearance and/or species life-history traits, remains poorly understood. We quantified the concentrations of the two key eggshell pigments, protoporphyrin IX and biliverdin, from a diverse sample of eggshells stored at the Natural History Museum, Tring, UK. We explicitly tested how these two pigments are associated with physical measures of eggshell coloration and whether the pigment concentrations and colour diversity co-vary with phylogenetic affiliations among species. We also tested a series of comparative hypotheses regarding the association between the concentrations of the two pigments and specific life-history and breeding ecology traits. Across species, the average concentrations of protoporphyrin and biliverdin were positively correlated, and both strongly co-varied with phylogenetic relatedness. Controlling for phylogeny, protoporphyrin concentration was associated with a higher likelihood of cavity nesting and ground nesting, whereas biliverdin concentration was associated with a higher likelihood of non-cavity nesting habit and bi-parental provisioning. Although unlikely to be explained by a single function, the breeding ecology and life history-dependence of eggshell pigment concentrations in these comparative analyses implies that related species share pigment strategies, and that those strategies relate to broad adaptive roles in the evolution of variation in avian eggshell coloration and its underlying mechanisms. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **106**, 657–672.

ADDITIONAL KEYWORDS: biliverdin – phylogenetically controlled GLM – protoporphyrin.

INTRODUCTION

From the glossy bright blue egg of the great tinamou (*Tinamus major*) to the calligraphic lines on a common murre (*Uria aalge*) egg, the striking colours and maculation of avian eggs continue to inspire humans both scientifically (Cassey *et al*., 2010b) and aesthetically (Purcell, Hall & Corado, 2008). Identifying the causes and functions of colourful phenotypic variation is a principal model system for understanding the evolution of trait diversity in modern evolutionary ecology (Hubbard *et al*., 2010). Yet, the varied

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appearance of bird eggs constitutes a poorly understood aspect of avian reproductive investment (Wallace, 1889; Kilner, 2006; Zimmerman & Hipfner, 2007). Among birds, the majority of species share similar (white) background eggshell colours (Cassey *et al.*, 2010b). Where variability in visible eggshell coloration does exist, it is believed to be aligned with differences that typically correspond with variation in the presence and concentration of the two key tetrapyrrole pigments (protoporphyrin IX and biliverdin) responsible for avian eggshell coloration across diverse avian lineages (Kennedy & Vevers, 1976; Gorchein, Lim & Cassey, 2009).

The comparative patterns of the evolutionary causes, the functional roles, and the ecological and life-history traits associated with colourful eggshell appearance have been reviewed in detail (Underwood & Sealy, 2002; Kilner, 2006; Cherry & Gosler, 2010), and no single hypothesis seems adequate to explain the adaptive significance of variability in colourful eggshell appearance among wild birds (Reynolds, Martin & Cassey, 2009). It is most likely that eggshell pigmentation evolved as a trade-off between its role as a structural component of the physical calcite barrier, protecting the needs of the developing embryo within the shell (reviewed by Maurer, Portugal & Cassey, 2011), and the visual appearance of the individual eggs. Regarding visible aspects of eggshell appearance, pigmentation may be selected to achieve crypsis (e.g. avoidance of predation; Tinbergen *et al.*, 1962), mimicry (e.g. by brood parasites; Davies & Brooke, 1989), or different strategies of conspicuousness (e.g. Birkhead, 1978; Davies, 2000; Lyon, 2003; Hanley, Doucet & Dearborn, 2010). Whether the pigments themselves represent a costly maternal trade-off of the maternal investment of the reproducing female (e.g. Moreno & Osorno, 2003; Moreno *et al.*, 2006; Hanley & Doucet, 2009; Avilés, Soler & Hart, 2011) is currently hotly debated (Riehl, 2011). Surprisingly, how concentrations of eggshell pigments vary among related species and how this variability is associated with either eggshell appearance or species biology (i.e. life history and reproductive ecology) remain unknown (Sparks, 2011).

Considerable research effort has focused on the basic biochemistry of avian eggshell pigments (Kennedy & Vevers, 1976; Mikšík, Holan & Deyl, 1996; Gorchein *et al.*, 2009), and the presence of porphyrins and related bile pigments was identified over a century ago (Sorby, 1875; Fischer & Kögl, 1923; Lemberg, 1934). The two key tetrapyrrole pigments (protoporphyrin IX and biliverdin) are involved in the synthesis and catabolism of haem (Milgrom, 1997), both circulate in the bloodstream, and are metabolized *de novo* in the shell gland (Poole, 1965; Wang *et al.*, 2009). The presence and absence of pyrrole pigments in eggshells of over 100 species of extant bird (Kennedy & Vevers, 1976), and three extinct species (Igic *et al.*, 2010), has already been documented. Still, without quantification of their concentrations it has been difficult to test evolutionary and adaptive roles of these pigments in the appearance and coloration of avian eggshells across phylogenetic lineages and different scales of evolutionary and ecological diversity.

To assess the extent to which the proximate mechanisms generating the apparent diversity of avian eggshell appearance are shared (or differ) across close and distant evolutionary lineages, we quantified the concentration of protoporphyrin IX and biliverdin from a diverse sample of eggshells laid by Neoaves birds breeding in Britain. We tested whether these two pigments are associated with physical measures of eggshell coloration and how pigment concentrations and colour diversity predictably co-vary among species across several levels of phylogenetic relatedness. Based on predictions of recent reviews of avian eggshell colour diversity (Underwood & Sealy, 2002; Kilner, 2006; Cherry & Gosler, 2010), we also evaluated a series of comparative hypotheses regarding the association between the concentrations of the two pigments and specific life-history and breeding ecology traits of the sampled species set. Specifically, we tested whether higher concentrations of both pigments will be detected in species that do not nest in cavities and/or nest in open environments (i.e. ground- and open cup-nesting species), whose egg appearance might (1) be more easily perceived, to aid in the putative signalling functions of crypsis (Wallace, 1889); and/or (2) be used in blackmailing co-incubating mates to protect from predation (Hanley *et al.*, 2010); as well as (3) subserve the possible physical benefits of protection from ultraviolet light (Lahti, 2008) and/or thermoregulation (Bertram & Burger, 1981). In addition, the pigment protoporphyrin IX alone is hypothesized to have a possible structural function (Gosler, Higham & Reynolds, 2005) and is therefore predicted to compensate for physical shell strength weakened due to other factors (e.g. possibility of low-calcium diet and the resulting thinner eggshells; but see Maurer *et al.*, 2011). Finally, the pigment biliverdin alone is predicted to be associated with costly maternal investment through its antioxidant properties, owing to possible trade-offs between reproductive investment and self-maintenance (Moreno & Osorno, 2003; Moreno *et al.*, 2004; Morales, Velando & Moreno, 2008; Morales, Velando & Torres, 2011). Accordingly, we tested whether biliverdin concentration is positively associated with traits involved in the length and timing of the reproductive investment and the intensity of parental care (e.g. the length of the incu-

bation and fledging periods, clutch size, and occurrence of bi-paternal care; but see Krist & Grim, 2007; Walters & Getty, 2010).

MATERIAL AND METHODS

Eggshell samples

Eggshells were made available for chemical analyses through a destructive loan of breeding bird (Neoaves) species of the British Isles from scientific material stored at the Natural History Museum, Tring, UK. These eggs all constitute so-called 'shoebox' collections from private collectors and are lacking the quality of data required for accession to the main collection (see Russell *et al*., 2010). In particular, data for date and locality are insufficient to include as co-variates in a quantitative (historical or geographical) analysis. For 49 avian genera, the species with the greatest number of egg sets available was chosen and three eggs were randomly sampled per species from different collections (i.e. most likely to differ in clutch identity, date, and location) and of 'good' or superior quality (as recorded by Russell *et al*., 2010). Natural History Museum accession numbers are provided for each of the samples (Supporting Information, Appendix S1).

Whole eggs were washed in de-ionized water to remove any external dirt, and dried at room temperature for 48 h. A fragment (surface area > 1 cm^2) was cut from the equatorial region of the shell (Fig. S1) using a Microtorque 2 dental drill (Milnes Bross., Croydon, UK) with an 817T diamond head (Intensive Swiss Dental, Grancia, Switzerland). Fragment thickness was measured to an accuracy of 1 μm using a Mitutoyo Series 227-203 constant measurement force micrometer (following Maurer *et al*., 2011). Both anvils of the micrometer were custom-fitted with 6-mm aluminium pins (diameter 1.35 mm) with rounded tips of 0.675-mm radius. Fragments were placed in the micrometer so that they were at 90° to the pin and measured at three different locations across the fragment at a measurement force of 1.5 N.

Pigment extraction and quantification

Protoporphyrin IX and biliverdin were quantified in the form of their dimethylesters (following Mikšík *et al*., 1996). Pigments were extracted and esterified in absolute methanol (15 mL; LiChrosolv, gradient grade for chromatography, Merck, Darmstadt, Germany) containing concentrated sulphuric acid (5%) at room temperature in the dark under N_2 for 24 h. Extract solutions were decanted and chloroform (10 mL; Merck; chloroform GR, ISO) and distilled water (10 mL) were added, and the mix was then shaken. The lower (chloroform) phase was collected, and the upper (aqueous) phase was again extracted with chloroform (chloroform phases from both extractions were collected). These phases were washed in 10% NaCl (5 mL), followed by distilled water until the wash solution was neutral. Extracts were evaporated to dryness and reconstituted in chloroform (1 mL) with an internal standard [5,10,15,20-tetra(4-pyridyl)-21H,23H-porphine, Sigma-Aldrich, St Louis, MO, USA; 0.01 mg mL^{-1}]. Commercially sourced standards for quantification (protoporphyrin IX and biliverdin, MP Biomedicals, LLC, Eschwege, Germany) were treated by the same procedure.

Pigments were identified and their concentration was quantified by reversed-phase high-performance liquid chromatography (HPLC) using an Agilent 1100 LC system (Agilent, Palo Alto, CA, USA). Chromatographic separation was conducted in a Gemini 5u C18 110A column (250 × 2.0 mm inner diameter, Phenomenex, Torrence, CA, USA). The sample (10 μL) was injected and eluted in a column with a gradient consisting of (A) methanol–water–pyridine, 35:65:0.25 v/v, and (B) methanol–acetonitrile–pyridine 90:10:0.25 v/v (flow rate 0.3 mL min^{-1}, 55 °C). The gradient started at A/B 80:20 reaching 10:90 after 15 min and after 10 min it reached 100% B. For the next 10 min the elution was isocratic at 100% B. Elution was monitored by absorbance at 410 nm and by fluorescence at 405/620 nm (excitation/emission).

Pigment detection and quantification were confirmed by the same HPLC which was coupled to an ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra; Agilent). Elution was achieved with a linear gradient (A = water with 0.1% formic acid, and B = acetonitrile with 0.085% formic acid), at a flow rate of 0.35 mL min^{-1} and at 55 °C. The gradient started at A/B 80:20 reaching 10:90 after 15 min and reaching 100% B after 5 min. For the next 10 min the elution was isocratic. We used atmospheric pressure ionization-electrospray ionization positive mode ion-trap mass spectrometry with the multiple reaction monitoring mode when precursor ions were 619 (internal standard), 611 (biliverdin), and 591 (protoporphyrin IX).

Operating conditions were: drying gas (N_2), 12 L min^{-1}; drying gas temperature, 350 °C; nebulizer pressure, 30 p.s.i. (207 kPa); with the elution monitored by absorbance at 410 nm.

Pigment concentration

Following previous studies, the concentration of the pigments was standardized by the mass (g^{-1}) of the eggshell sample fragments (Mikšík *et al*., 1996; Moreno *et al*., 2006). Shell fragments were weighed to an accuracy of 0.001 g on a Mettler PC 440 digital scale. This measure of concentration (nmol g^{-1}) is suitable to standardize the measurement of concen-

tration if pigment deposition occurs throughout the entire depth of the eggshell matrix (e.g. Nys *et al.*, 2004; Jagannath *et al.*, 2008).

However, at least one study has reported that the majority of pigmentation may occur in the outermost layer (Wang *et al.*, 2007), and therefore a better measure of pigment concentration might be standardized by the surface area of the sample fragment (mm^{-2}) (Igic *et al.*, 2010). Eggshell fragments were therefore photographed using a Canon EOS 450D digital camera with a 105-mm Sigma AF lens. This camera was mounted on a Kaiser camera stand enclosed within two Calumet photographic umbrellas lined with silver-white (AU3046) and flat white (AU3045) lining. Samples were illuminated with two OSRAM 11-W energy-saving light bulbs producing a light of a colour temperature of 6000 K to the right and front of the sample. Photographs were taken at ISO 400 and aperture of f16, while exposure varied from 0.2 to 6.0 s depending on the brightness of each species' egg. Each fragment was photographed twice: first, against a black velvet (photographic standard) background; and second, against a white technical 2×2-mm grid. The surface area of the flat eggshell fragments was estimated from the area covered on the grid surface. We calculated the Pearson's correlation coefficient between pigment concentrations when standardized for fragment mass or for surface area, and in all subsequent analyses we included both measures of pigment concentration to evaluate any differences in standard statistical significance and interpretation.

Nested analysis of variance was conducted in SAS v9.2 (SAS Institute Inc., Cary, NC, USA) to determine the size (percentage) of the component of variance, in pigment concentration, attributed to the independent replicate eggs (within a species), compared with that among species and families. Species and families were classified following the taxonomy of Monroe & Sibley (1997).

Sample validation

We have documented, previously, significant differences in a range of spectral reflectance-based coloration-metrics between eggshells stored in museums and freshly collected eggs (Cassey *et al.*, 2010a, 2011). These differences are probably due to temporal change in chemical structure of the pigments through storage exposure to light, and/or different preservation techniques. The pigments biliverdin and protoporphyrin have both been successfully extracted from eggshell fragments > 650 years old of extinct bird species (Igic *et al.*, 2010), and thus may constitute a little perishable type of pigmented biological structure. To assess the potential influence that time in storage (i.e. photo-oxidation) may have on pigment concentrations used in our analyses, we compared pigment concentrations for two species (*Larus ridibundus* and *Turdus philomelos*) of five museum eggs, each egg from a different clutch, with ten fresh eggshell samples opportunistically collected from other studies (Cassey *et al.*, 2008; Maurer *et al.*, 2011). Museum samples were all donated to the NHM between 1890 and 1950. Although these eggshells may have experienced different opportunities for photo-oxidation, as a group we expect them to be sufficiently different from fresh samples that differences in pigment concentrations will be clearly apparent if they indeed exist. The fresh samples were collected prior to the onset of incubation (i.e. before visible embryo development) and prepared for pigment analyses in the same manner as described above. The average concentrations for each of the two pigments were compared using unequal variance *t*-tests in SAS v9.2 (SAS Institute).

Colorimetry of sample eggshell colour

Modern digital photography is a powerful method for measuring the relationships between multiple patches of biological coloration (Pike, 2011). Quantification of chromaticity using photography requires that the chosen camera gives a consistent estimate of the chromaticies present within that sample (Stevens *et al.*, 2007); these estimates need to be consistent between photographs to yield 'relative consistency'. It is also desirable that photographs capture accurately the true chromaticities of the photographed sample to yield 'absolute consistency'. All digital eggshell images were photographed against a black velvet photographic standard background (see above) and saved in standardized RAW format. RAW images are beneficial for colour analyses for two reasons: first, this format has no spatial compression and a larger range of pixel brightness values (12–14 bit); and second, in RAW-format images, white-balancing operations are not applied to the digital image but are merely saved alongside the image within the same file. So, with the appropriate software, it is possible to recover linear uncompressed digital images.

The theory and detailed process of characterizing and calibrating for the spectral sensitivities of a digital camera have been summarized previously (Lovell *et al.*, 2005). The target for the polynomial mapping of linearized RGB triplets values were the CIE XYZ colour-space coordinates (CIE, 1986). Once all of the images were converted from RGB values to XYZ, conversion from XYZ to CIELUV space (see Fig. 1) was implemented using the Matlab image processing toolbox (2008a, The MathWorks, Natick, MA, USA). The CIELUV colour space was chosen, first, because it approaches perceptual uniformity (for

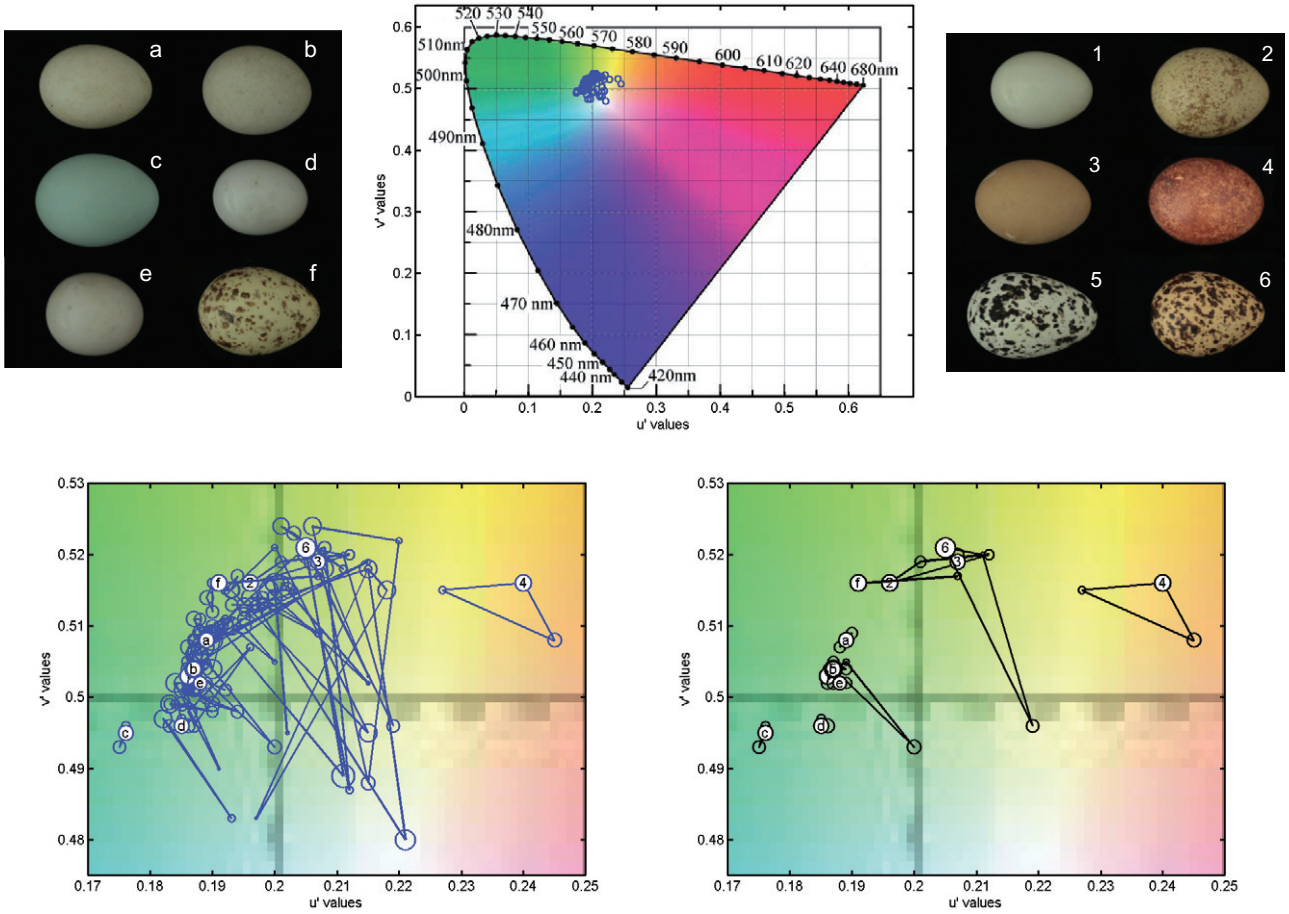

Figure 1. The CIELUV chromaticity space occupied by the average of the three principal colours for each of the 49 species' eggs ($n = 3$ eggs per species) is provided in the top central panel. In the bottom two panels, the three principal colours for a single different egg from each species are joined by straight (nearest distance) lines, with the size of the points denoting the proportion of pixel coverage each colour contributes. In the bottom right panel, only the 12 eggs pictured are presented. The species are: (a) *Haliaeetus albicilla*, (b) *Circus aeruginosus*, (c) *Ardea cinerea*, (d) *Alcedo atthis*, (e) *Merops apiaster*, (f) *Larus ridibundus*; (1) *Picus viridis*, (2) *Alectoris rufa*, (3) *Botaurus stellaris*, (4) *Falco peregrinus*, (5) *Alca torda*, (6) *Tringa totanus*. Note that the immaculate eggs (e.g. a, c, d) share very similar colours compared with the maculated eggs (e.g. f, 5, 6). Lightness (or saturation) is not represented in this figure.

human observers), so changes of the same magnitude along the three axes should be similarly visible. Secondly, CIELUV values are achieved with a mathematically simple transformation from XYZ. In this colour space, the L' channel corresponds to lightness (a correlate of saturation; Wyszecki & Stiles, 1982), and the u' and v' channels correspond to red/green and blue/yellow opponent values, respectively.

The predominant sample colour was determined using a k-means clustering procedure and assuming three predominant colours within the sampled region (see Fig. 1). We based our choice of three predominant colours on the two known pigments and their interaction (absence or presence). We used the MIXED procedure in SAS v9.2 (SAS Institute) to construct multivariate regression models, with species identity as a repeated random effect, and tested the influence of the increased concentration (see Pigment concentration) of the two pigments (protoporphyrin IX, biliverdin) as explanatory variables with the response Y implemented as an n-by-3 matrix of the CIELUV variables (L', u', v') for the first predominant colour.

The degree of maculation (spotting) present in an eggshell sample's photograph was estimated as the ratio of the foreground and background regions using greyscale thresholding, with the threshold at which to segment determined automatically in Matlab using the Otsu clustering thresholding process (Otsu, 1979). We assumed that the darker regions were the foreground 'spots' and this was visually confirmed in all cases. The foreground-to-background ratio was calculated as the number of pixels in the foreground region

divided by the number of pixels in the background region (average 0.17; range 0.00–0.97).

Comparative life-history and breeding ecology traits

Life-history data were gathered primarily from the *Birds of the Western Palearctic* (Cramp & Simmons, 1978–1994) and cross referenced with the *Handbook of the Birds of the World* Volumes 1–7 (del Hoyo, Elliott & Christie, 1992–2002) and from family- and species-specific monographs. Arithmetic midpoints were used in cases where a range of values was presented. Information on clutch size, incubation length and fledging period, nest location (ground, tree/cliff, or cavity) was taken from the species-specific descriptions. These references were used to determine whether provisioning is shared between the sexes, or performed by the female or male alone, and whether developmental mode was altricial versus not altricial (i.e. eyes open or closed at hatching; Ricklefs, 1968). Species with a high-calcium diet were contrasted with those with low-calcium diets. For each species, the whole eggs (as different from sample fragments) were assessed by three independent observers for presence and coverage of maculation using a three-point scoring system (Kilner, 2006). Maculation was recorded for each egg as 0 – if the egg was immaculate, 1 – for maculation present but with a clear, dominant background colour, and 2 – for widespread maculation that covered the majority of the egg. It was intended that an average score would be calculated across observers, but the three observers were in agreement in all cases.

Phylogenetic hypotheses and tests

We revised and updated a recent phylogenetic hypothesis of British birds (Thomas, 2008). The Thomas phylogeny was based on sequence data from 12 protein-coding mitochondrial genes and included 248 British breeding bird species. We improved this tree by: (1) adding sequence data for 15 more species, (2) increasing the number of genes included for many species, and (3) replacing data on Thomas's three surrogate species with recently published data on the focal species (little bittern *Ixobrychus minutus*, European bee-eater *Merops apiaster*, and European golden plover *Pluvialis apricaria*). Each gene was aligned by eye in SE-AL v. 2.0a11 (Rambaut, 2002). All sequence data were collected from GenBank (Benson *et al*., 2008) using Geneious v. 4.8.5 (Drummond *et al*., 2010). Sequence accessions and full alignments are available from the authors on request. We used BEAST 1.5.4 for phylogenetic analyses using a codon-specific GTR + Γ substitution model in which substitution rates, among-site rate variation, and state frequencies at third codon positions were unlinked ($GTR + CP_{112} + \Gamma$). We used a Yule prior on the branching process and an uncorrelated relaxed clock in which rate variation among branches was drawn from a log-normal distribution. We applied two topology constraints to the phylogeny by defining the monophyly of the widely accepted Neoaves and Galloanserae clades. Note that this is more liberal than the 11 constraints used by Thomas (2008) and allows us to better account for the uncertainty in topology in the deeper nodes of avian phylogeny. We conducted two runs for 40 and 50 million generations, respectively, sampling trees every 10 000 generations. We assessed mixing within runs and convergence between runs using Tracer v. 1.5.0 (Rambaut & Drummond, 2007) based on visual inspection of traces and effective sample sizes of tree parameters (node ages of the two constrained nodes), posterior log-likelihoods and substitution model parameters. Both runs converged rapidly and we discarded 10% of generations from each run as burnin. We combined the post-burnin samples of the two runs to yield a posterior distribution in which the majority of parameters had effective sample sizes > 500 (and all > 100). For use in subsequent phylogenetic analyses (see below) we sub-sampled down to 1000 trees (drawn from the full posterior distribution of > 8000 trees) and pruned each tree to the 49 species in the eggshell data set. We also extracted the maximum clade credibility (MCC) tree from the full tree distribution for use as a single 'best' representative tree.

As a measure of the strength of phylogenetic signal in the pigment concentration variables we estimated Pagel's lambda, λ (Pagel, 1999; Freckleton, Harvey & Pagel, 2002), using the R-library motmot (available from http://r-forge.r-project.org/). 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 covariances between species for a given trait (Freckleton *et al*., 2002). To assess the effects of phylogenetic error, we repeated the λ fitting procedure to a distribution of 1000 phylogenetic hypotheses (see above).

We tested hypotheses on the correlates of pigment concentration using the R-library CAIC to fit phylogenetically controlled general linear models. Specifically, we used the function pglmEstLambda to fit Pagel's lambda simultaneously with each regression model in order to appropriately correct for phylogenetic signal in the residuals. We first tested the correlation between the two pigment concentration variables without any other covariates and repeated this over 1000 phylogenies. We then fitted full regression models including all relevant explanatory

variables with the concentration of biliverdin and protoporphyrin IX respectively as response variables. From the full models we simplified the model by backwards removal of each non-significant explanatory variable in turn. We also added each removed variable back into the reduced model one by one to assess model robustness. The initial full model and model simplification were conducted on the MCC tree only. To assess the robustness of parameter estimates and significance to phylogenetic uncertainty we subsequently ran the simplified model across all 1000 phylogenetic hypotheses. Results are presented as means ± 1 standard error. The tabulated chemical and comparative data is provided as an Appendix (supporting Appendix S1).

RESULTS

Sample validation

The fresh concentration of the two pigments (nmol g^{-1}) differed between the two species with *L. ridibundus* eggshell being predominant in protoporphyrin (2.6×), and *T. philomelos* eggshell being predominant in biliverdin (3.1×). There was no significant difference in the concentration of protoporphyrin between fresh and museum eggshell samples for *L. ridibundus* (mean ± standard error of difference = 0.055 ± 0.117; $t = 0.56$, d.f. = 11.99, $P = 0.589$) or *T. philomelos* (0.194 ± 0.215; $t = 0.77$, d.f. = 5.67, $P = 0.472$). There was no significant difference in the concentration of biliverdin between fresh and museum eggshell samples of *T. philomelos* (0.127 ± 0.398; $t = 0.64$, d.f. = 10.24, $P = 0.538$) or *L. ridibundus* (0.627 ± 0.197; $t = 2.59$, d.f. = 4.97, $P = 0.061$).

Pigment concentration and eggshell appearance

The average sample concentrations, standardized either for weight (g^{-1}) or for surface area (1000 mm^{-2}), were highly correlated across species for both pigments (Pearson's correlation coefficients $r > 0.96$, $n = 49$; Fig. 2). Across species, the average concentration of protoporphyrin and biliverdin was positively correlated (Fig. 3).

An increase in an average measure of the maculation of the eggshell sample (the ratio of foreground pattern with respect to background shell colour) was significantly associated with an increase in both protoporphyrin ($\log_{10}$) concentration (slope ± SE = 0.144 ± 0.021, $R^2 = 0.51$, $P < 0.001$) and biliverdin ($\log_{10}$) concentration (0.112 ± 0.035, $R^2 = 0.18$, $P = 0.003$). In a multivariate regression model, differences in CIELUV (L', u', v') colour

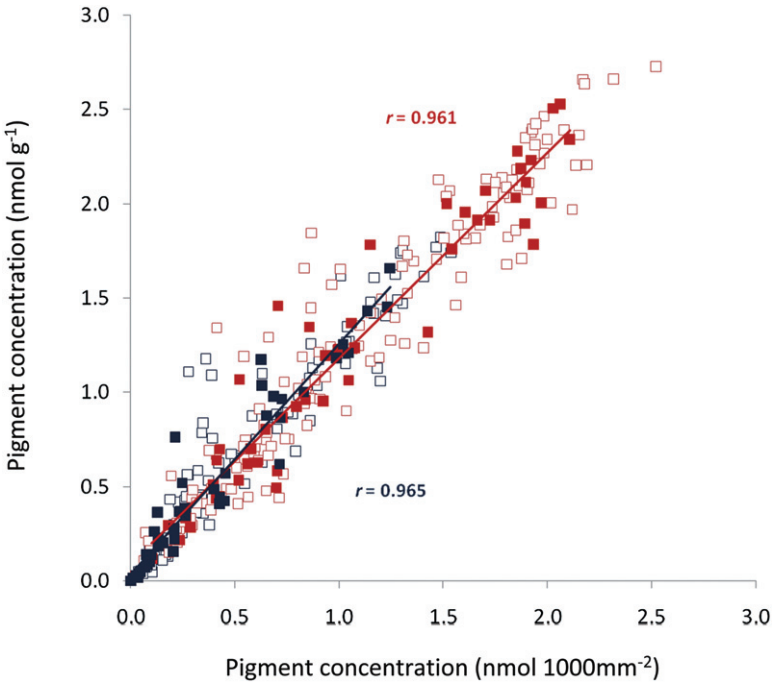

Figure 2. Bivariate scatterplot of the positive association between ($\log_{10}$) pigment concentrations standardized by fragment sample surface area (mm^{-2}) and fragment sample weight (g^{-1}) for protoporphyrin IX (red loci) and biliverdin (blue loci). Open squares indicate values for individual fragments (three per species). Lines of best fit were estimated by ordinary least squares regression and are fitted through the 49 species means (solid loci).

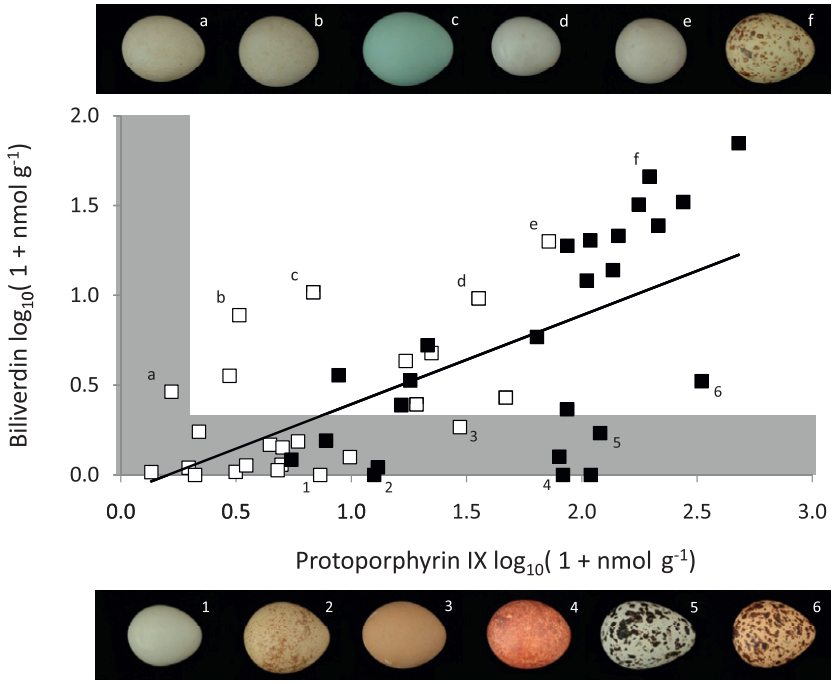

Figure 3. Bivariate scatterplot of the interspecific relationship between the average concentration [$\log_{10}(1 + \text{nmol g}^{-1})$] of the eggshell pigments protoporphyrin IX and biliverdin. Species with maculated (patterned) eggshells (closed squares) or with immaculate eggshells (open squares) are distinguished. The shaded (grey) region of the graph indicates values less than 1 nmol g^{-1}. The line of best fit is estimated by ordinary least squares regression (Pearson's correlation $r = 0.803$, $n = 49$, $P < 0.001$). Species with high residuals across the relationship (chosen non-randomly) are depicted for both protoporphyrin IX (1–6) and biliverdin (a–f). In all cases the photographs (taken by G.M.) are of an actual egg used in subsequent analyses prior to the removal of a shell fragment. The species are: (1) *Picus viridis*, (2) *Alectoris rufa*, (3) *Botaurus stellaris*, (4) *Falco peregrinus*, (5) *Alca torda*, (6) *Tringa totanus*; (a) *Haliaeetus albicilla*, (b) *Circus aeruginosus*, (c) *Ardea cinerea*, (d) *Alcedo atthis*, (e) *Merops apiaster*, (f) *Larus ridibundus*.

Table 1. Results of a multivariate regression model testing the influence of increasing pigment concentration (nmol g^{-1}) on measures of CIELUV colour space response variables (L', u', v') for the predominant colour-averaged variable

Effect	Estimate (SE)	d.f.	t	P
Protoporphyrin IX				
L'	–0.207 (0.048)	49	–4.30	< 0.001
u'	0.011 (0.002)	49	4.81	< 0.001
v'	0.006 (0.002)	49	3.44	0.001
Biliverdin				
L'	0.049 (0.064)	49	0.76	0.454
u'	–0.008 (0.003)	49	–2.66	0.010
v'	–0.003 (0.002)	49	–1.07	0.290

space coordinates were significantly associated with increases in the concentration of both pigments, for the predominant coloration of the eggshell (Table 1). Notably, an increase in protoporphyrin concentration was associated with a decrease in lightness (L') and a positive change in u' and v' colour coordinates. In contrast, an increase in biliverdin concentration was only significantly associated with a negative change in u' (Table 1).

Phylogenetic patterns in eggshell coloration

For the 49 British breeding bird species analysed here, the percentage of variance (nested analysis of variance) in pigment concentration attributed to within species (among replicate shell samples from different collections) was approximately 15% for both pigments (Table 2).

The degree of phylogenetic relatedness (Pagel's λ) was significantly different from zero for both pigments, but not significantly different from one (Table 3). These results were robust to phylogenetic uncertainty and consistent across 1000 re-sampled phylogenetic trees (see Methods). Controlling for phylogenetic relatedness, the interspecific positive correlation between average concentration of protoporphyrin and biliverdin was highly significant (Fig. 4). This positive correlation was consistent across all of the 1000 re-sampled trees. Across species

Table 2. Results from a nested analysis of variance to determine the component of variance (percentage) in eggshell pigment concentration, attributed to the three replicate eggs (within a species), compared with the total variance among different species (within higher taxa – levels not shown)

		Protoporphyrin IX (nmol g^{-1})		Biliverdin (nmol g^{-1})		Protoporphyrin IX (nmol 1000 mm^{-2})		Biliverdin (nmol 1000 mm^{-2})	
	d.f.	Variance	Per cent	Variance	Per cent	Variance	Per cent	Variance	Per cent
Total	146	0.573		0.296		0.448		0.187	
Among replicate eggs (within species)	98	0.084	14.65	0.047	15.78	0.066	14.79	0.029	15.31

Table 3. The degree of phylogenetic dependence (Pagel's λ) calculated for pigment concentration, as the maximum-likelihood estimate of the multiplier of the off-diagonal elements of the variance–covariance matrix implied by the phylogeny (following Freckleton *et al*., 2002)

	Pagel's λ	Max likelihood	LR test = 0	LR test = 1
		(nmol g^{-1})		
Protoporphryin IX	1.00	–40.01	25.97***	0
Biliverdin	1.00	–30.67	16.08***	0
		(nmol 1000 mm^{-2})		
Protoporphryin IX	0.89	–15.37	10.13***	0.24
Biliverdin	0.99	–32.89	25.74***	0

The likelihood ratio (LR) values are given for the model with Pagel's λ set to 0 and to 1 (equivalent to a standard general linear model). The significance of maximum likelihood values of Pagel's λ (χ^2 critical value, d.f. = 1, $\alpha = 0.05$) was calculated using a likelihood ratio test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

(controlling for phylogenetic relatedness), an increase in the concentration of one of the pigments (protoporphyrin or biliverdin) was always associated with a greater concentration of the other (Table 4).

Phylogenetic models of eggshell coloration

Across species, as we initially predicted, increased protoporphyrin concentration was consistently associated with a higher propensity of maculated shell patterning, whereas biliverdin concentration was consistently associated with a higher propensity of immaculate shells (Table 4). Protoporphyrin concentration was associated with a higher likelihood of cavity nesting (tree-hole and burrow) compared with non-cavity nesting, and a higher likelihood of ground nesting compared with tree or cliff nesting (Table 4). In turn, biliverdin concentration was associated with a higher likelihood of non-cavity-nesting habit (compared with burrow and tree-hole nesting), and a greater likelihood of bi-parental provisioning (Table 4).

DISCUSSION

A number of recent reviews of the diverse eggshell appearance in birds have all concluded that eggshell pigmentation has evolved under multiple selection pressures operating simultaneously to increase embryo survival in different ways (Underwood & Sealy, 2002; Kilner, 2006; Reynolds *et al*., 2009; Cherry & Gosler, 2010). Such diverse sets of avian eggshell appearances and selection pressures have led to the suggestion that looking for general patterns of eggshell coloration may be less than worthwhile, if not altogether impossible (Kilner, 2006). Mechanistically, the underlying sources of the diversity in avian eggshell appearance are two ubiquitous pigments (Milgrom, 1997). By contrast, other animal calcite structures (e.g. mollusc shells) exhibit a great diversity of pigments, including many different porphyrins, and a range of other pigment classes to match their wide variation in coloration and appearance (Comfort, 1951; Bandaranayake, 2006).

Pigment concentrations co-vary with eggshell appearance, both in colour and maculation

The comparative scope of our study was only possible through the unique material available to us through the scientific collection of the Natural History Museum, Tring, UK (Russell *et al*., 2010). By utilizing this material on bird species from a single biogeo-

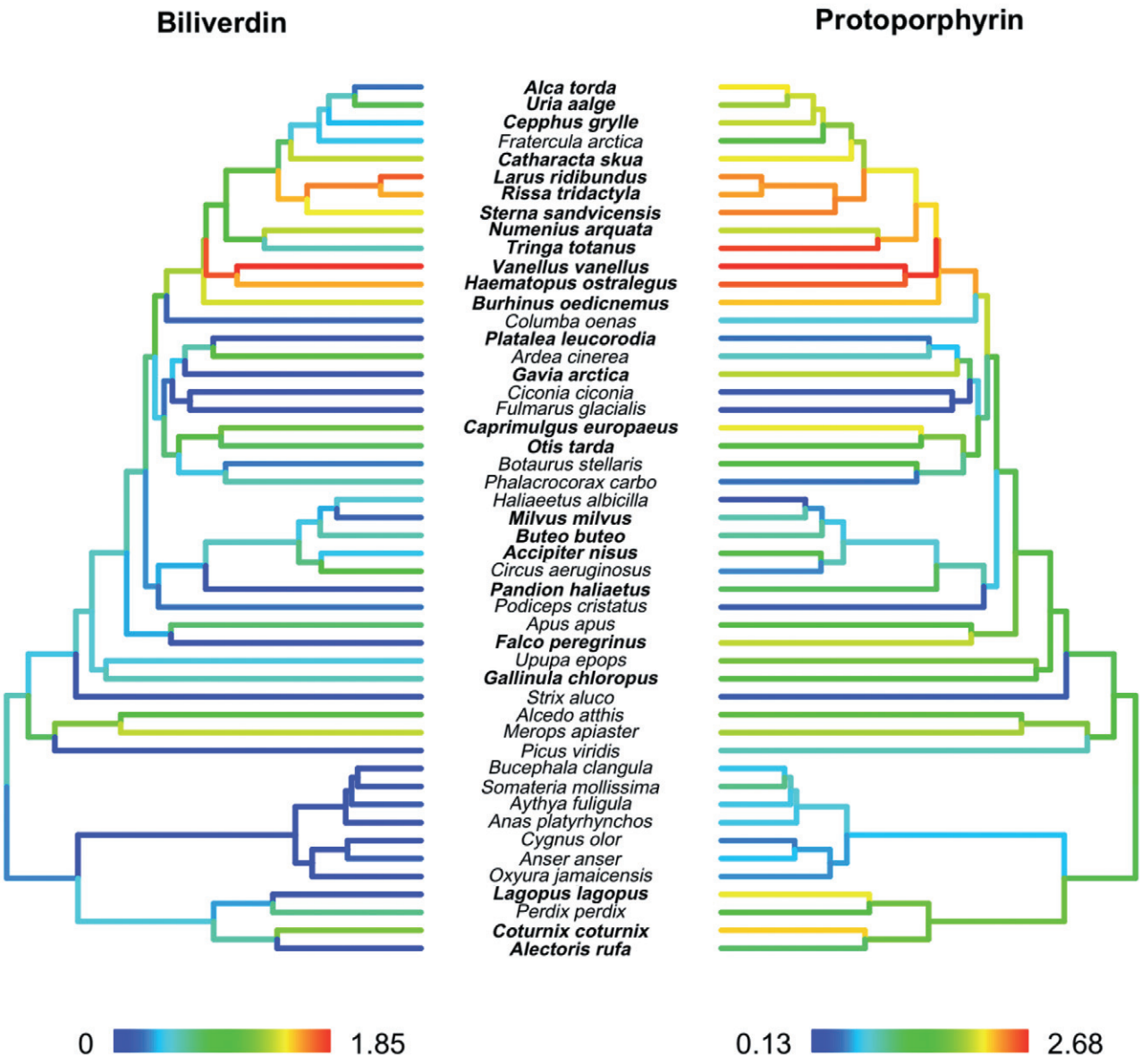

Figure 4. Maximum likelihood consensus phylogenetic tree for 49 species used in the comparative analysis of the association between species biology and eggshell pigment concentration. The coloured branches depict the concentration [$\log_{10}(1 + \text{nmol g}^{-1})$] of the two pigments protoporphyrin IX and biliverdin. Species names with maculated (patterned) shells are labelled in bold. The phylogenetic correlation between the two pigments is positive and highly significant (see Results).

graphical region, we were able to directly study the proximate mechanisms generating the apparent diversity of avian eggshell appearance across broad evolutionary lineages. However, there were several a priori reasons to assume that museum eggshells would differ in their pigment concentration from fresh eggshells. Pigment-based appearance, with coloration measured as reflectance spectra, changes during incubation (Moreno, Lobato & Morales, 2011), with climatic factors (Avilés *et al*., 2007), and with time since collection (Starling *et al*., 2006; Cassey *et al*., 2010a, 2011). It is therefore interesting that no significant differences were detected between pigment concentrations of independent fresh and museum eggshells from two very different species – a gull (dominant in protoporphyrin) and a thrush (dominant in biliverdin). Less surprising is that in all cases the direction of the effects was as predicted if detectable intact pigment concentration was expected to decrease with time (fresh concentration > museum concentration), and in one case it indeed approached conventional statistical significance (biliverdin in *L. ridibundus*).

Table 4. PGLS models for the maximum-likelihood phylogenetic hypothesis test of variables putatively associated with increases in the concentration (nmol g^{-1}) of the eggshell pigments protopohyrin IX and biliverdin

	Full model			Final model			
	Estimate (SE)	*t*	*P*	Estimate (SE)	*t*	*P*	Trees
Protoporphyrin IX	Adjusted $R^2 = 0.630$			Adjusted $R^2 = 0.787$			
(Intercept)	1.516 (0.368)	4.124	0.000	1.451 (0.185)	7.828	0.000	
Biliverdin $\log_{10}$(nmol g^{-1})	0.420 (0.131)	3.208	0.003	**0.596 (0.103)**	**5.776**	**0.000**	1000
Maculation (0, 1)	0.557 (0.089)	6.276	0.000	**0.600 (0.073)**	**8.187**	**0.000**	1000
Eggshell thickness (nm)	–1.628 (0.521)	–3.127	0.003	–0.878 (0.390)	–2.254	0.029	1000
Incubation length (days)	–0.009 (0.010)	–0.915	0.366				
Fledging period (days)	0.004 (0.003)	1.417	0.165				
Clutch size (eggs per brood)	–0.029 (0.027)	–1.056	0.298				
Cavity type (none vs. burrow/tree hole)	0.240 (0.153)	1.568	0.126	**0.497 (0.157)**	**3.167**	**0.003**	860
Nest type (ground vs. tree/cliff)	–0.146 (0.139)	–1.049	0.301	**–0.359 (0.118)**	**–3.037**	**0.004**	878
Parental care (bi- vs. uni-)	0.027 (0.190)	0.143	0.887				
Development (altricial vs. not altricial)	–0.139 (0.174)	–0.796	0.431				
High calcium diet (0, 1)	–0.062 (0.118)	–0.523	0.604				
Biliverdin	Adjusted $R^2 = 0.560$			Adjusted $R^2 = 0.616$			
(Intercept)	–0.034 (0.462)	–0.073	0.942	–0.061 (0.198)	–0.307	0.760	
Protoporphyrin IX $\log_{10}$(nmol g^{-1})	0.589 (0.144)	4.095	< 0.001	**0.633 (0.092)**	**6.869**	**0.000**	1000
Maculation (0, 1)	–0.456 (0.132)	–3.461	0.001	**–0.425 (0.099)**	**–4.278**	**0.000**	1000
Eggshell thickness (nm)	0.019 (0.671)	0.028	0.978				
Incubation length (days)	0.002 (0.012)	0.192	0.849				
Fledging period (days)	–0.009 (0.004)	–1.999	0.053	–0.006 (0.003)	–2.145	0.038	1000
Clutch size (eggs per brood)	–0.026 (0.028)	–0.933	0.357				
Cavity type (none vs. burrow/tree hole)	–0.493 (0.189)	–2.608	0.013	**–0.510 (0.136)**	**–3.734**	**0.001**	1000
Nest type (ground vs. tree/cliff)	0.091 (0.197)	0.464	0.646				
Parental care (bi- vs. uni-)	–0.321 (0.205)	–1.566	0.126	**–0.384 (0.120)**	**–3.195**	**0.003**	1000
Development (altricial vs. not altricial)	–0.211 (0.189)	–1.116	0.272				
High calcium diet (0, 1)	0.067 (0.144)	0.461	0.648				

Final models were confirmed by both forwards and backwards variable elimination and the contribution of changes to the model likelihood and AICc (corrected Akaike information criterion). The results presented are for the maximum clade credibility (MCC) tree. In addition, the number of trees (Trees) for which the variables in the final model are retained from 1000 randomly re-sampled phylogenies is also given. Variables in bold were retained (same direction of relationship) in models associated with an increase in pigment concentration, standardized by fragment surface area (1000 mm^{-2}). In no cases were any additional terms retained.

We therefore make the conservative conclusion that it is likely that pigment concentrations may decrease with storage, and this decrease would be predicted to be statistically significant with larger sample sizes (or older samples), but the effect size is still not as great as would be a priori expected based on patterns of physical and perceivable colour changes with time in storage (Cassey *et al*., 2011). Because of the nature of the samples used we were unable to accurately record year of collection (see Methods), or include it as a covariate in the analyses. However, all of the eggs used in the interspecific analyses were chosen from species with a large number of samples available and randomly selected from different collections with the highest quality available (as independently recorded by Russell *et al*., 2010). Notably, there is no evidence that differential effects (i.e. interactions) between pigment types and species biased our results.

In general, for our sample of British breeding birds, an increase in protoporphyrin was associated with a decrease in u' and increase in v' colour space coordinates and a decrease in lightness, i.e. less saturated colours that are more likely to be perceived as red–brown–yellow. Furthermore, biliverdin was associated with an increase in u', i.e. colours that are more likely to be perceived as blue–green. These observations fit reasonably well with previous predictions that protoporphyrin and biliverdin are likely to manifest themselves as red–brown and blue–green pigments, respectively (Kennedy & Vevers, 1976). Nevertheless, the variability we detected in pigment concentrations is considerable and simple predictions regarding the

relative contributions of the two pigments based on differences in coloration are likely to be highly misleading (e.g. see Fig. 3). For example, if we compare the eggs of *Picus viridis* (Fig. 3, 1) and *Ardea cinerea* (Fig. 3, c) then the concentration of protoporphyrin is similar but that of the biliverdin is different and the egg of *Ardea cinerea* is visibly more blue, as would be expected. However, comparison of these two species with the eggs of *Alcedo atthis* (Fig. 3, d) and *Merops apiaster* (Fig. 3, e), both with higher concentrations of biliverdin and protoporphyrin, have visibly white eggs rather than maculated as might be predicted. Although it is well known that pigments can be present in visibly white eggs, this is the first time that inter-specific relationships between coloration and eggshell pigmentation have been quantified. Interestingly, the difficulty with making simple visually based assumptions for estimating eggshell pigment concentration is similar to what has been previously reported for feather colours, and pigment quality and quantity, in birds (McGraw *et al*., 2004).

Phylogenetic patterns in eggshell pigmentation

The emergence of the field of comparative biology offers a means of testing quantitative hypotheses on the evolution of biochemical eggshell pigmentation (Felsenstein, 1985). We found that the concentrations of the two pigments are positively correlated across species, and that both pigments also exhibit strongly co-varying phylogenetic patterns. Within a species it is known that the concentration of protoporphyrin and biliverdin can be positively correlated (Wang *et al*., 2009; Dongxiang hens *Gallus g. domesticus*, $r = 0.97$, $n = 30$, $P < 0.01$). Yet, it is interesting that this positive correlation, between the two pigments, also occurs interspecifically across such a diverse range of eggshell appearances and bird species. Wang *et al*. (2009) proposed that the two pigments are possibly derived from the same precursor metabolic pathway. In contrast, it had been assumed that protoporphyrin IX was a precursor to biliverdin (Needham, 1974) and so an increase in the concentration of one of the two pigments would be predicted to be most likely linked to a decrease in the other. Clearly, there remains a number of interesting questions unanswered regarding the production and deposition of both these pigments, including the biological and measurable costs of putative trade-offs in maternal investment to wild female birds (Morales *et al*., 2008).

Cherry and Gosler (2010) concluded that the most convincing explanation for the primary function of protoporphyrin maculation is a structural compensation for eggshell thinning. Accordingly, we found that for a measure of protoporphyrin concentration (nmol g^{-1}), its increase was significantly associated with thinner eggshells across species. Yet, an alternative explanation for this result is that thicker average eggshell fragments weigh more (positive correlation between average shell thickness and fragment weight; $r = 0.97$, $n = 49$, $P < 0.001$), and this measure of the concentration of pigment could be subsequently biased if pigmentation occurs mostly in the surface cuticle layer of the shell (e.g. Wang *et al*., 2007). Indeed, despite the two measures of pigment concentration (per sample fragment weight and surface area) being highly correlated (Fig. 2) it is apparent that, in our analyses, a general relationship between eggshell thickness and protoporphyrin is dependent on the specific calculation of pigment concentration (Table 4), and that considerable care will be needed to resolve the biological relevance of this statistical relationship.

Protoporphyrin-based eggshell coloration

As predicted, protoporphyrin pigment concentration was consistently associated with species that lay maculated eggshells, and was higher in ground-nesting species. That protoporphyrin is deposited more by species with egg coloration and maculation more visible through open nesting (e.g. ground nesting) supports a possible signalling function, specifically crypsis from predators (Weidinger, 2001). Subsequently, it is more surprising that greater protoporphyrin pigmentation is also consistently (and independently) associated with cavity-nesting species. Specifically, this finding raises the interesting question of whether the pigment protoporphyrin IX plays a role independent of perceivable signalling (i.e. crypsis) through modifying eggshell appearance, and perhaps subserves physical functions including modifying gas conductance through the shell matrix critical for embryonic development (e.g. Higham & Gosler, 2006) and/or providing antimicrobial protection to the shell (Hincke, Nys & Gautron, 2010; Ishikawa *et al*., 2010). In these cases, the prediction would be that increased protoporphyrin is associated with nesting habits that are particularly humid and susceptible to increased microbial infection (Beissinger, Cook & Arendt, 2005; Cook *et al*., 2005), as supported by our data.

Biliverdin-based eggshell coloration

In contrast to protoporphyrin concentration, the life-history traits with which biliverdin pigmentation was consistently associated were a non-cavity (open cup but not ground) nesting habit, and an increased propensity for bi-parental provisioning. Differences in

the coloration of eggshells between cavity- and open-nesting birds have been long considered in the published literature (Lack, 1948; Götmark, 1992; Kilner, 2006), and we have demonstrated that variation in eggshell pigments is indeed responsible for these differences. Higher concentrations of biliverdin in open-nesting and bi-parental birds supports a number of recently developed signalling and physical hypotheses (reviewed in Reynolds *et al*., 2009). However, given repeatedly inconsistent experimental and correlational support for some of these hypotheses, we recommend caution when interpreting this result and strongly encourage more species-specific studies to understanding the function and evolution of biliverdin-based pigmentation for eggshell coloration. Despite speculation about the role of biliverdin as a hydrophilic antioxidant in birds it is not justified to assume a priori that functional antioxidant properties of biliverdin are present in eggshells (Riehl, 2011). Whereas recent work has investigated the dietary (Morales *et al*., 2011) and hereditary (Morales *et al*., 2010) components of eggshell pigmentation, and their interaction, studies confirming the specific antioxidant roles (and costs) of circulating versus deposited biliverdin in birds are urgently required. It is clear that this direction of research deserves further attention, and will indeed be greatly rewarding.

SUMMARY

Our results have revealed that the concentrations of eggshell pigments are more phylogenetically conserved than either qualitative or quantitative studies of shell colour would have us believe (Kilner, 2006; Cassey *et al*., 2010b). In addition, pigment concentrations are phylogenetically associated with different key ecological and life-history strategies. We have highlighted a number of fascinating future directions for the study of eggshell pigmentation in wild birds. Of particular importance will be determining the genes responsible for egg coloration (Fossøy *et al*., 2011; Wang *et al*., 2011), and understanding their role in the production of haem (Wang *et al*., 2009). It has been reported that differences in pigment deposition can be related to indicators of female quality and stress (Morales, Sanz & Moreno, 2006; Mertens *et al*., 2010), and it remains to be determined whether pigment deposition represents a costly trade-off to the laying female (Morales *et al*., 2011). We believe that future work that strives to understand the physiological mechanisms underlying eggshell pigmentation will be of the greatest interest. There is no doubt that this will prove both challenging and rewarding to future evolutionary and mechanistic studies of avian reproductive investment.

ACKNOWLEDGEMENTS

For assistance with museum samples we are particularly grateful to Douglas Russell, Judy White, and the staff at the Natural History Museum, Tring, UK. We thank John Allen and Dan Hanley, as well as three anonymous reviewers, for comments that considerably improved a previous version of this manuscript. Funding was provided by the Leverhulme Trust Fund (to P.C. and M.E.H.), and the Human Frontier Science Program RGY69/07 (to P.C., M.E.H. and T.G.). P.C. is an ARC Future Fellow.

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SUPPORTING INFORMATION

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

Figure S1. Eggshell fragments ($> 1\ cm^2$) were cut from the equatorial region of the whole shell. Digital photographs were taken of each sample and a binary mask was constructed to locate the eggshell sample in the photograph for all subsequent colorimetric analyses.

Appendix S1. NHM accession numbers and life history comparative data for all species.

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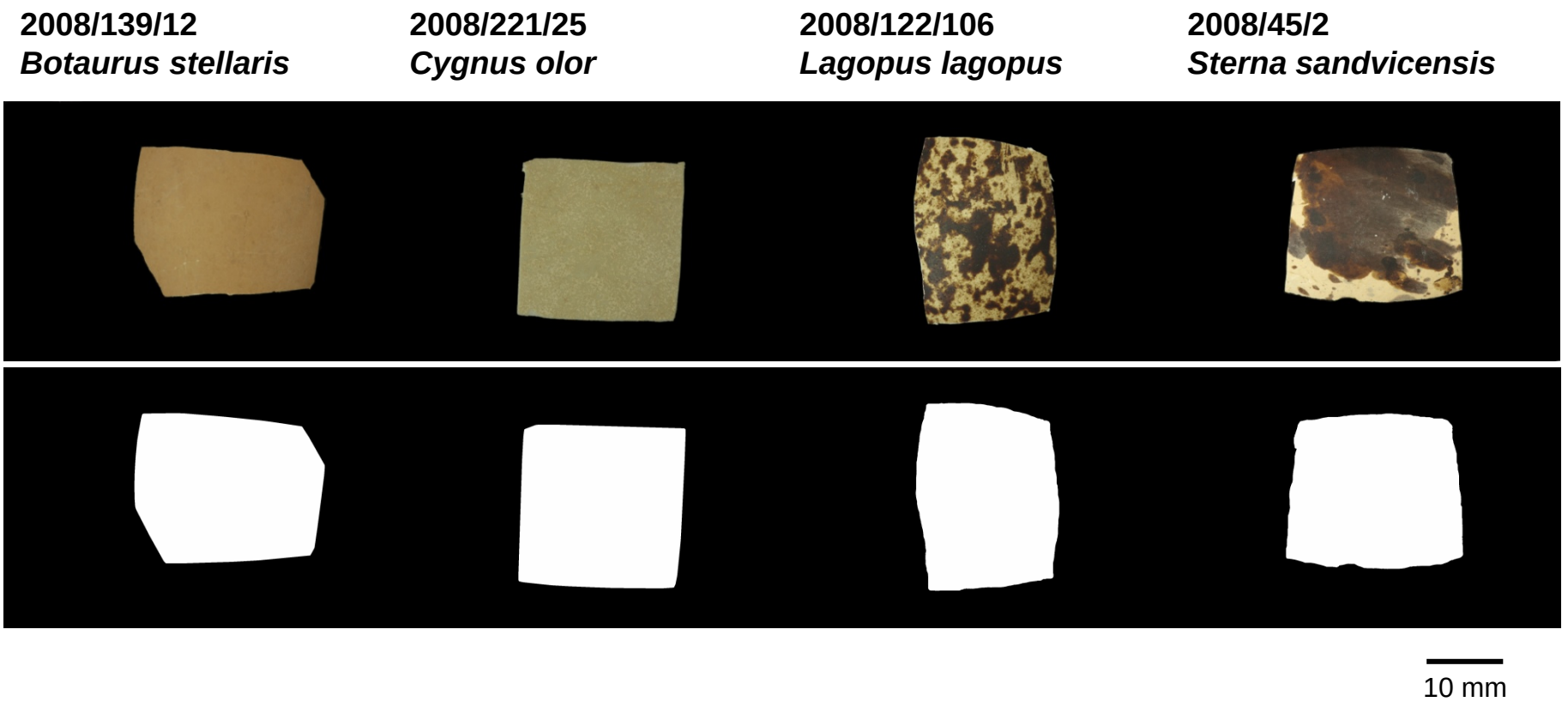
Figure S1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2008/221/25
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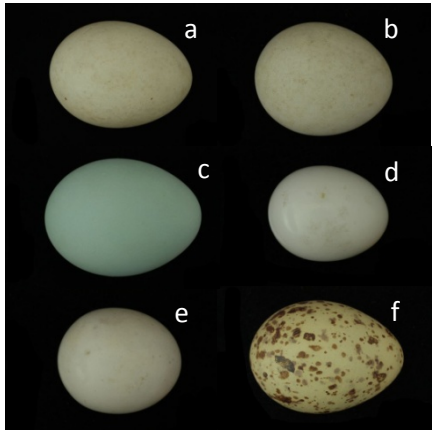
2008/122/106
Lagopus lagopus
2008/45/2
Sterna sandvicensis
10 mm

Figure 1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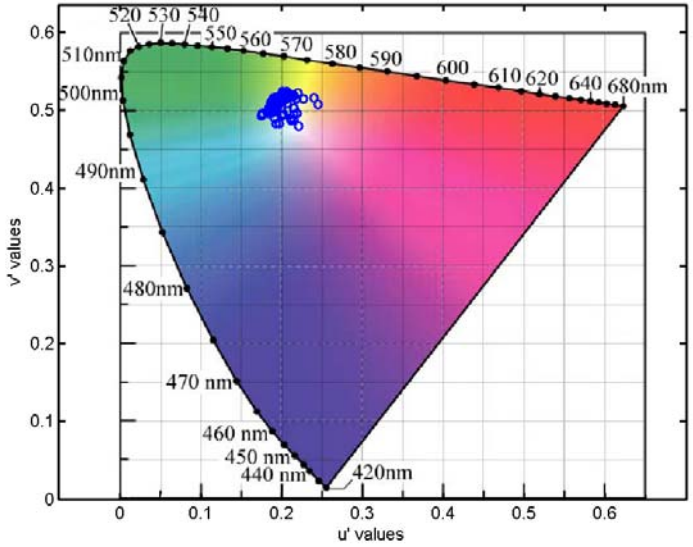
b
c
d
e
f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500nm
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460 nm
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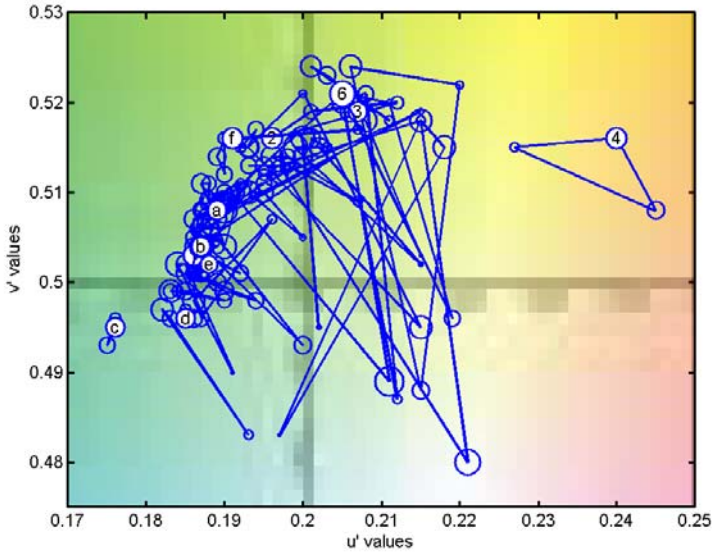
420nm
v' values
u' values


1
2
3
4
5
6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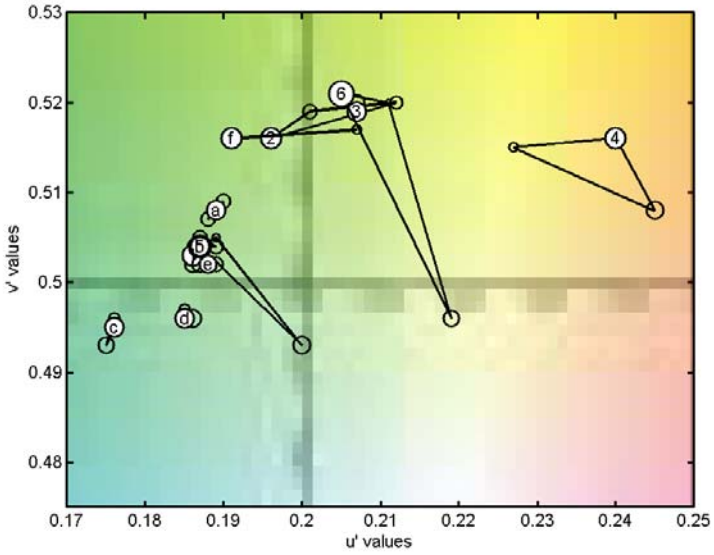
v' values
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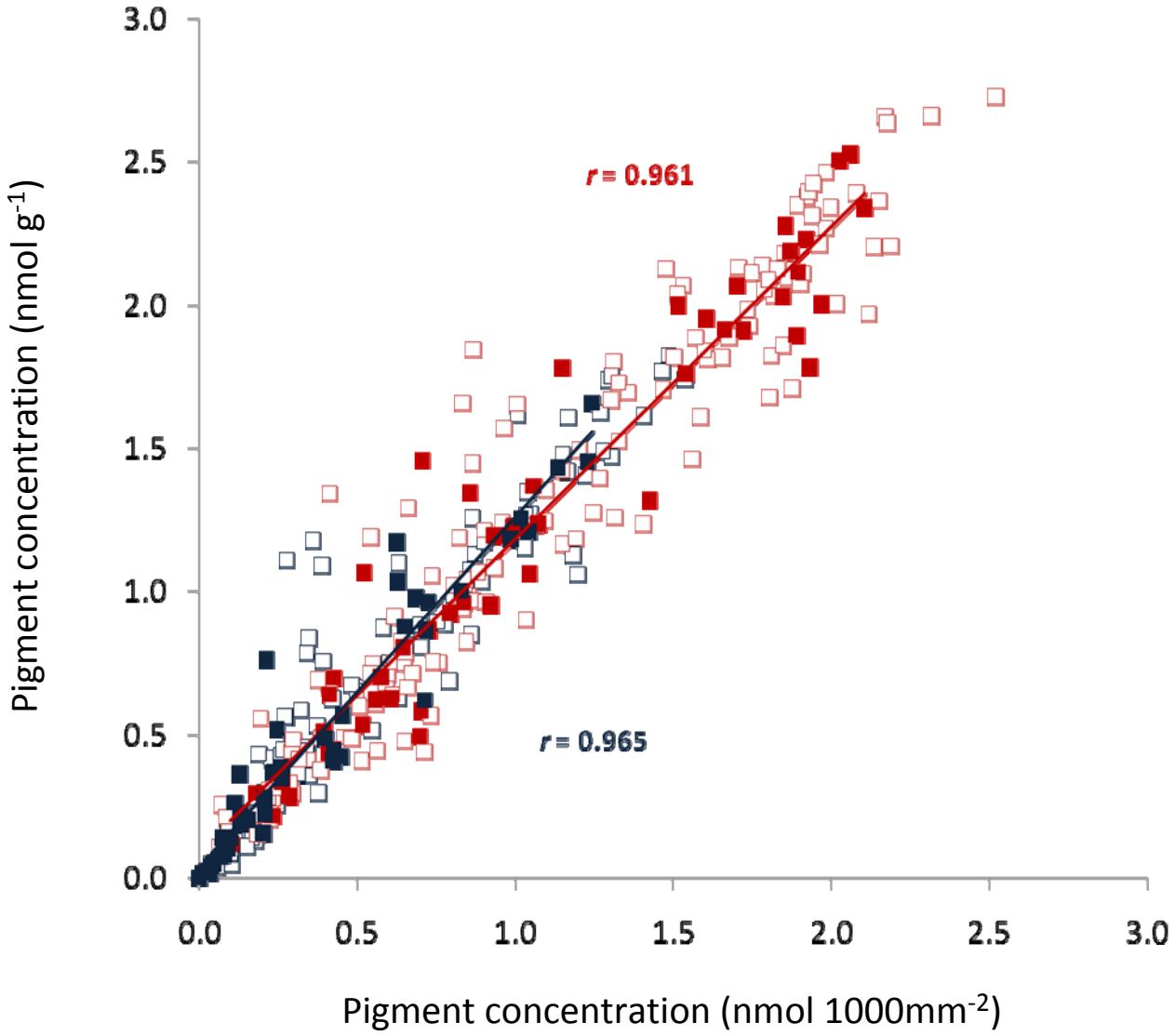
Figure 2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2.0
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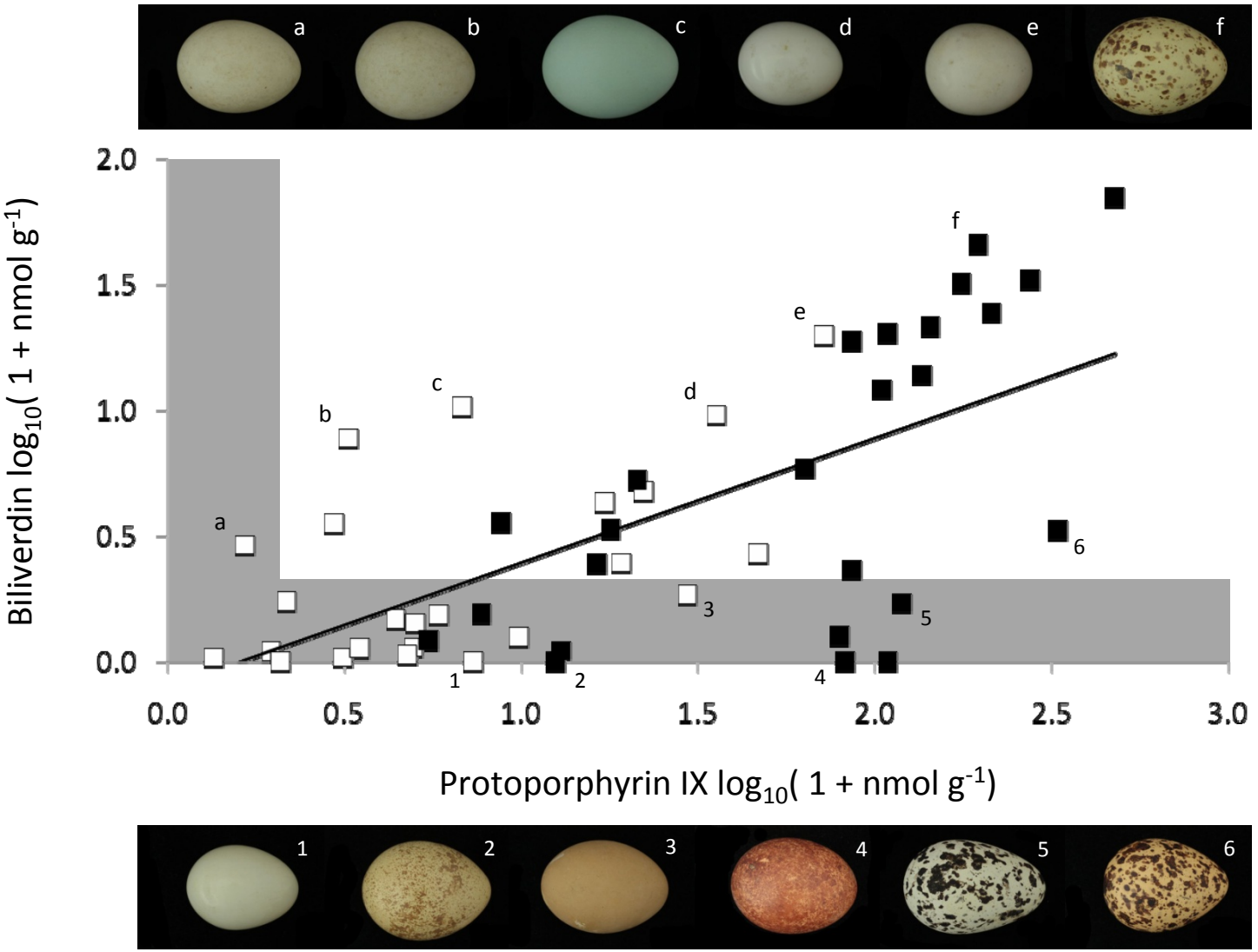
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
r = 0.961
r = 0.965
Pigment concentration (nmol g-1)
Pigment concentration (nmol 1000mm-2)

Figure 3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b
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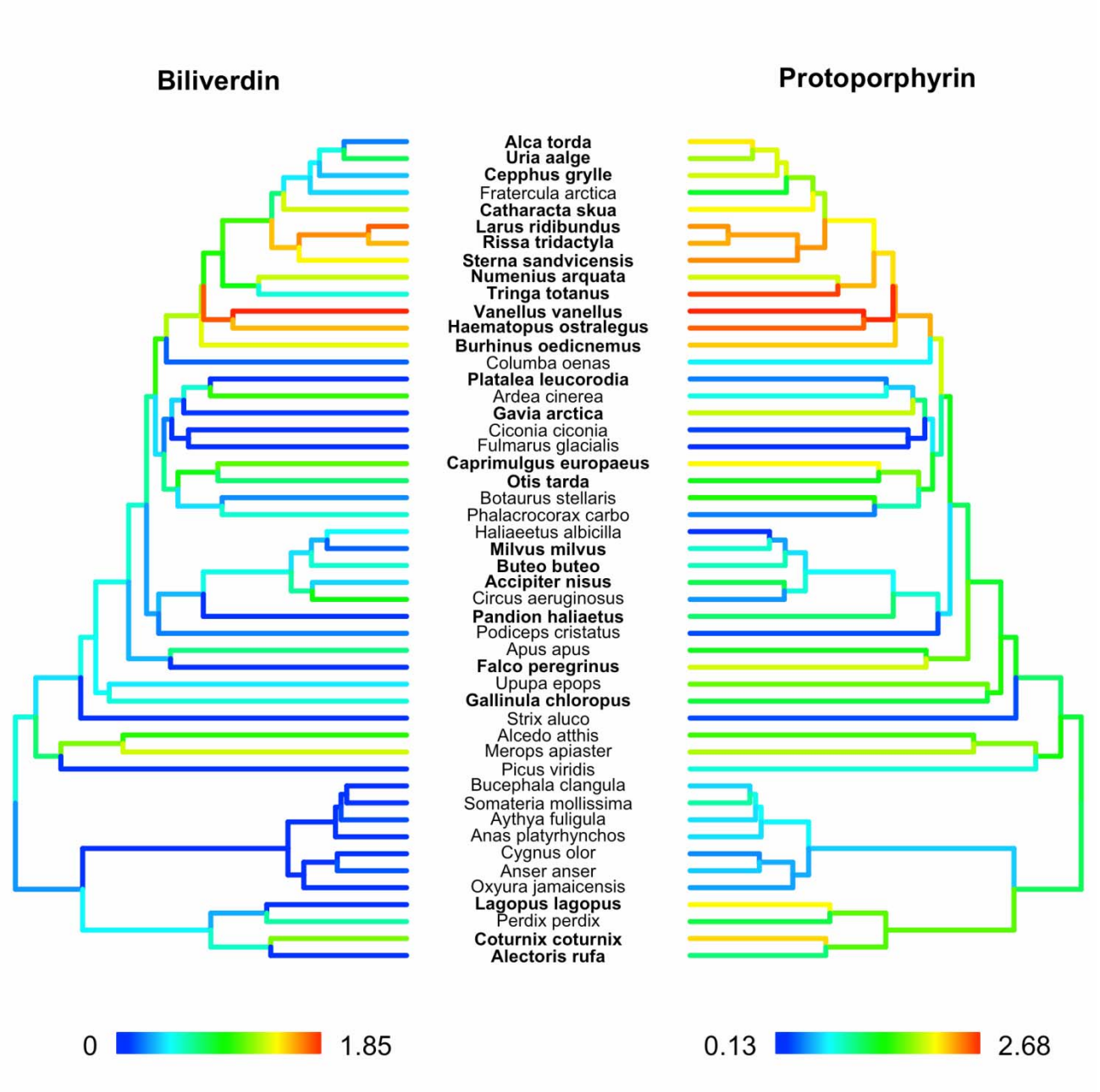
Biliverdin log10(1 + nmol g-1)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Protoporphyrin IX log10(1 + nmol g-1)
1
2
3
4
5
6

Figure 4


Biliverdin
Protoporphyrin
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Uria aalge
Cepphus grylle
Fratercula arctica
Catharacta skua
Larus ridibundus
Rissa tridactyla
Sterna sandvicensis
Numenius arquata
Tringa totanus
Vanellus vanellus
Haematopus ostralegus
Burhinus oedicnemus
Columba oenas
Platalea leucorodia
Ardea cinerea
Gavia arctica
Ciconia ciconia
Fulmarus glacialis
Caprimulgus europaeus
Otis tarda
Botaurus stellaris
Phalacrocorax carbo
Haliaeetus albicilla
Milvus milvus
Buteo buteo
Accipiter nisus
Circus aeruginosus
Pandion haliaetus
Podiceps cristatus
Apus apus
Falco peregrinus
Upupa epops
Gallinula chloropus
Strix aluco
Alcedo atthis
Merops apiaster
Picus viridis
Bucephala clangula
Somateria mollissima
Aythya fuligula
Anas platyrhynchos
Cygnus olor
Anser anser
Oxyura jamaicensis
Lagopus lagopus
Perdix perdix
Coturnix coturnix
Alectoris rufa
0
1.85
0.13
2.68

Figure legends

Figure S1. Eggshell fragments (> 1cm ^{2}) were cut from the equatorial region of the whole shell. Digital photographs were taken of each sample and a binary mask was constructed to locate the eggshell sample in the photograph for all subsequent colorimetric analyses.

Figure 1. The CIELUV chromaticity space occupied by the average of the three principal colours for each of the 49 species' eggs (n = 3 eggs per species) is provided in the top central panel. In the bottom two panels, the three principal colours for a single different egg from each species, are joined by straight (nearest distance) lines, with the size of the points denoting the proportion of pixel coverage each colour contributes. In the bottom right panel, only the twelve eggs pictured are presented. The species are: (a) *Haliaeetus albicilla*, (b) *Circus aeruginosus*, (c) *Ardea cinerea*, (d) *Alcedo atthis*, (e) *Merops apiaster*, (f) *Larus ridibundus*; (1) *Picus viridis*, (2) *Alectoris rufa*, (3) *Botaurus stellaris*, (4) *Falco peregrinus*, (5) *Alca torda*, (6) *Tringa totanus*. Note that the immaculate eggs (e.g., a, c, d) share very similar colours compared with the maculated eggs (e.g., f, 5, 6). Lightness (or saturation) is not represented in this figure.

Figure 2. Bivariate scatterplot of the positive association between pigment concentrations standardized by fragment sample surface area (mm^{-2}) and fragment sample weight (g^{-1}) for protoporphyrin IX (red loci) and biliverdin (blue loci). Hollow loci indicate values for individual fragments (3 per species). Lines of best fit were estimated by ordinary least squares regression and are fitted through the 49 species means (solid loci).

Figure 3. Bivariate scatterplot of the interspecific relationship between the average concentration ($\log_{10}(1 + \text{nmol } g^{-1})$) of the eggshell pigments protoporphyrin IX and biliverdin. Maculated (patterned) species (solid loci) and immaculate species (hollow loci) are distinguished. The shaded (grey) region of the graph indicates values less than one nmol g^{-1}. The line of best fit is estimated by ordinary least squares regression (Pearson's correlation $r = 0.803$, $n = 49$, $P < 0.001$). Species with high residuals across the relationship (chosen non-randomly) are depicted for both protoporphyrin IX (1-6) and biliverdin (a-f). In all cases the photographs (taken by GM) are of an actual egg used in subsequent analyses prior to the removal of a shell fragment. The species are: (1) *Picus viridis*, (2) *Alectoris rufa*, (3) *Botaurus stellaris*, (4) *Falco peregrinus*, (5) *Alca torda*, (6) *Tringa totanus*; (a) *Haliaeetus albicilla*, (b) *Circus aeruginosus*, (c) *Ardea cinerea*, (d) *Alcedo atthis*, (e) *Merops apiaster*, (f) *Larus ridibundus*.

Figure 4. Maximum likelihood consensus phylogenetic tree for 49 species used in the comparative analysis of the association between species biology and eggshell pigment concentration. The coloured branches depict the concentration (log10) of the two pigments protoporphyrin IX and biliverdin. Species names with maculated (patterned) shells are labelled in **bold**. The phylogenetic correlation between the two pigments is positive and highly significant (see Results).

Family	Genus_species	NHM Accession numbers	Biliverdin (nmol.g-1)	Protoporphyrin (nmol.g-1)	Maculation (0,1,2)	Eggshell_thickness (mm)	Incubation_length (days)	Fledging_period (days)	Clutch_size (eggs.brood -1)	Cavity_type	Nest_type	Parental_provisioning	Development	High_Calcium_Diet (0, 1)
Phasianidae	Alectoris_rufa	2008/27/2; 2008/68/3; 2008/72/2	0.00	11.56	2	0.28	24.0	10.0	12.0	none	ground	uniparental	not-altricial	0
Phasianidae	Perdix_perdix	2001/102/1; 2007/31/21; 2008/98/10	3.31	16.22	0	0.24	24.0	15.0	16.0	none	ground	uniparental	not-altricial	0
Phasianidae	Coturnix_coturnix	2007/109/3A; 2006/45/26A; 2008/199/28A	12.82	135.46	1	0.15	18.5	19.0	7.0	none	ground	uniparental	not-altricial	0
Phasianidae	Lagopus_lagopus	2008/122/106; 2008/32/73; 2008/140/19	0.00	108.38	2	0.21	24.0	12.5	12.0	none	ground	uniparental	not-altricial	0
Anatidae	Oxyura_jamaicensis	2006/45/3; 2008/72/344; 2008/60/1	0.13	2.50	0	0.44	24.0	52.5	7.0	none	ground	uniparental	not-altricial	1
Anatidae	Cygnus_olor	2008/221/25; 2008/138/100; 2008/72/339	0.04	2.15	0	0.78	36.0	135.0	6.0	none	ground	biparental	not-altricial	0
Anatidae	Anser_anser	2008/72/338; 2008/50/129; 2007/109/38	0.47	3.44	0	0.67	27.5	55.0	5.0	none	ground	biparental	not-altricial	0
Anatidae	Anas_platyrhynchos	2008/13/6; 2008/50/129; 2008/30/3	0.14	3.98	0	0.31	27.5	55.0	11.0	none	ground	uniparental	not-altricial	0
Anatidae	Aythya_fuligula	2008/35/6; 2008/50/120; 2008/13/21	0.42	4.03	0	0.32	35.0	47.5	9.5	none	ground	uniparental	not-altricial	0
Anatidae	Somateria_mollissima	2008/122/105; 2008/50/128; 2006/45/4	0.26	8.86	0	0.37	26.5	70.0	4.0	none	ground	uniparental	not-altricial	1
Anatidae	Bucephala_clangula	2008/72/263; 2007/109/184; 2008/37/3	0.06	3.79	0	0.39	33.0	61.5	10.0	tree_hole	tree_cliff	uniparental	not-altricial	1
Picidae	Picus_viridis	2008/72/74; 2008/13/20; 2008/50/9	0.00	6.33	0	0.14	16.0	25.0	6.0	tree_hole	tree_cliff	biparental	altricial	0
Upupidae	Upupa_epops	2008/85/1; 2004/91/2a; 2008/95/11	1.69	45.80	0	0.11	18.0	27.5	6.5	tree_hole	tree_cliff	biparental	altricial	1
Alcedinidae	Alcedo_atthis	2008/142/2; 2008/122/81; 2008/119/11	8.62	34.69	0	0.09	20.0	20.0	6.5	burrow_cavity	tree_cliff	biparental	altricial	1
Meropidae	Merops_apiaster	2008/138/40B; 2008/122/25; 2008/111/36	18.97	70.94	0	0.11	21.0	22.5	5.5	burrow_cavity	tree_cliff	biparental	altricial	1
Apodidae	Apus_apus	2008/36/4; 2008/122/71; 2008/142/1	3.77	21.28	0	0.10	19.5	23.0	2.5	burrow_cavity	tree_cliff	biparental	altricial	1
Strigidae	Strix_aluco	2008/15/2; 2003/11/19; 2005/61/40	0.00	1.10	0	0.27	29.0	34.5	4.0	tree_hole	tree_cliff	biparental	altricial	1
Caprimulgidae	Caprimulgus_europaeus	2003/11/14; 2008/72/51; 2005/35/6	11.09	104.10	1	0.13	17.0	16.5	2.0	none	ground	biparental	altricial	1
Columbidae	Columba_oenas	2007/109/101; 2008/221/16; 2008/72/164	0.54	4.87	0	0.19	16.5	25.0	2.0	tree_hole	tree_cliff	biparental	altricial	0
Otididae	Otis_tarda	2008/50/131; 2008/139/1; 2008/105/1	4.27	20.42	1	0.56	26.5	32.5	2.0	none	ground	uniparental	not-altricial	0
Rallidae	Gallinula_chloropus	2008/74/4; 2008/30/4; 2007/113/3	2.36	17.02	1	0.25	20.5	45.0	7.0	none	ground	biparental	not-altricial	0
Scolopacidae	Numenius_arquata	2008/169/17; 2008/42/6; 2008/134/19	17.87	85.54	1	0.28	27.5	35.0	4.0	none	ground	biparental	not-altricial	1
Scolopacidae	Tringa_totanus	2007/109/93; 2008/119/4A; 2008/98/26	2.33	329.90	1	0.15	23.0	30.0	4.0	none	ground	biparental	not-altricial	1
Burhinidae	Burhinus_oedicnemus	2008/169/39; 2008/169/30; 2008/55/6	20.45	143.23	1	0.28	26.0	39.0	2.0	none	ground	biparental	not-altricial	1
Charadriidae	Haematopus_ostralegus	2008/32/69; 2008/72/294; 2005/61/41	32.08	274.29	2	0.27	27.0	30.0	3.0	none	ground	biparental	not-altricial	1
Charadriidae	Vanellus_vanellus	2008/32/90; 2008/98/43; 2008/30/5	69.28	478.06	1	0.19	26.0	37.5	4.0	none	ground	biparental	not-altricial	1
Laridae	Catharacta_skua	2008/55/60; 2008/15/7; 2008/17/6	19.21	107.81	1	0.33	29.0	45.5	2.0	none	ground	biparental	altricial	0
Laridae	Larus_ridibundus	2009/3/182; 2008/72/348; 2008/50/104	44.75	195.94	1	0.21	22.0	35.0	3.0	none	ground	biparental	not-altricial	0
Laridae	Rissa_tridactyla	2008/50/102; 2008/111/25; 2008/106/1	31.02	175.56	1	0.24	22.5	43.5	2.0	none	tree_cliff	biparental	not-altricial	1
Laridae	Sterna_sandvicensis	2008/15/4; 2008/45/2; 2008/32/71	23.48	213.77	1	0.23	22.5	29.0	2.0	none	ground	biparental	not-altricial	1
Laridae	Uria_aalge	2008/199/100A; 2008/209/4A; 2008/98/29	4.86	62.88	2	0.56	33.0	22.5	1.0	none	tree_cliff	biparental	altricial	1
Laridae	Alca_torda	2007/109/90; 2008/101/2; 2008/98/30	0.71	119.04	2	0.46	34.0	19.0	1.0	burrow_cavity	tree_cliff	biparental	altricial	1
Laridae	Cepphus_grylle	2008/32/84; 2008/25/6; 2008/98/15	1.32	85.30	1	0.32	28.0	41.0	1.0	burrow_cavity	tree_cliff	biparental	altricial	1
Laridae	Fratercula_arctica	2008/111/24; 2008/35/1; 2003/25/1	1.47	18.19	0	0.31	41.0	47.0	1.0	burrow_cavity	tree_cliff	biparental	altricial	1
Accipitridae	Pandion_haliaetus	2008/122/8; 2008/32/94; 2008/50/17	0.10	12.04	1	0.44	35.5	53.0	3.0	none	tree_cliff	biparental	altricial	1
Accipitridae	Milvus_milvus	2008/72/243; 2008/111/39; 2007/109/249	0.55	6.77	1	0.36	29.0	49.0	3.0	none	tree_cliff	biparental	altricial	1
Accipitridae	Haliaeetus_albicilla	2008/97/2; 2007/109/241; 2007/109/10	1.91	0.66	0	0.57	40.0	72.5	2.5	none	tree_cliff	biparental	altricial	1
Accipitridae	Circus_aeruginosus	2008/122/37; 2007/109/11a; 2008/95/42	6.76	2.27	0	0.32	32.0	37.5	3.5	none	ground	biparental	altricial	1
Accipitridae	Accipiter_nisus	2008/72/22; 2008/47/31; 2008/169/11	1.45	15.46	1	0.25	33.0	27.0	5.0	none	tree_cliff	biparental	altricial	1
Accipitridae	Buteo_buteo	2008/47/11; 2008/139/3a; 2008/169/22a	2.59	7.81	1	0.35	39.0	52.5	3.0	none	tree_cliff	biparental	altricial	1
Falconidae	Falco_peregrinus	2008/165/4; 2008/72/275; 2008/97/5	0.00	81.84	2	0.32	29.0	38.5	3.5	none	tree_cliff	biparental	altricial	1
Podicipedidae	Podiceps_cristatus	2008/169/26; 2008/13/53; 2008/98/8	0.74	1.18	0	0.30	27.0	75.0	4.0	none	ground	biparental	not-altricial	1
Phalacrocoracidae	Phalacrocorax_carbo	2008/105/4; 2003/11/3; 2008/72/241	2.56	1.96	0	0.43	29.0	50.0	3.0	none	tree_cliff	biparental	altricial	1
Ardeidae	Ardea_cinerea	2003/25/25; 2008/13/56; 2008/72/260	9.41	5.84	0	0.30	26.5	48.5	4.0	none	tree_cliff	biparental	altricial	0
Ardeidae	Botaurus_stellaris	2008/122/89; 2007/109/180; 2008/139/12	0.85	28.59	0	0.22	26.0	52.5	5.0	none	ground	uniparental	altricial	1
Threskiornithidae	Platalea_leucorodia	2008/50/115; 2008/140/31; 2008/111/17	0.22	1.93	1	0.41	24.5	47.5	4.0	none	tree_cliff	biparental	altricial	1
Ciconiidae	Ciconia_ciconia	2008/111/32; 2008/32/59; 2007/109/50	0.10	0.97	0	0.53	32.0	61.0	4.5	none	tree_cliff	biparental	altricial	0
Gaviidae	Gavia_arctica	2007/109/273; 2008/97/7; 2008/72/342	0.26	78.87	1	0.42	30.0	62.5	2.0	none	ground	biparental	not-altricial	1
Procellariidae	Fulmarus_glacialis	2008/50/132; 2008/98/20; 2008/72/336	0.04	0.36	0	0.39	50.0	48.5	1.0	none	tree_cliff	biparental	altricial	1