

## Research



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## Surface texture heterogeneity in maculated bird eggshells

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Many of the world's 10 000 bird species lay coloured or patterned eggs. The large diversity of eggshell patterning among birds, achieved through pigment, has been attributed to a few selective agents such as crypsis, thermoregulation, egg recognition, mate signalling, egg strength and protecting the embryo from UV. Pigmentation may influence the texture of eggshells, which in turn may be important for dealing with water and microbes. We measured surface roughness ( $S_{ar}$ , nm), surface skewness ( $S_{sk}$ ) and surface kurtosis ( $S_{ku}$ ), which describe different aspects of surface texture, across 204 bird species with maculated (patterned) eggs and 166 species with immaculate (non-patterned) eggs. Using phylogenetically controlled analyses, we tested whether maculated eggshells have different surface topography between the foreground colour and background colour, and between the background colour of maculated eggshells and the surface of immaculate eggshells. Secondly, we determined to what extent variation in eggshell pigmentation of the foreground and background colour is determined by phylogenetic relatedness, and whether certain life-history traits are important predictors of eggshell surface structure. We show that the surface of maculated eggs consists of a rougher foreground pigment compared to the background pigment across 71% of the 204 bird species (54 families) investigated. Species that lay immaculate eggs showed no difference in surface roughness, kurtosis or skewness compared to background pigment of maculated eggs. The difference in eggshell surface roughness between foreground and background pigmentation was greater among species that occupied dense habitats, such as forests with closed canopies, compared to those that nest in open and semi-open habitats (e.g. cities, deserts, grasslands, open shrubland and seashores). Among maculated eggs, foreground texture was correlated with habitat, parental care, diet, nest location, avian group and nest type, while background texture was correlated with clutch size, annual temperature, development mode and annual precipitation. Surface roughness among immaculate eggs was greatest for herbivores, and species that have larger clutch sizes. Together, this suggests that multiple life-history traits have influenced the evolution of eggshell surface textures in modern birds.

## 1. Introduction

The colour of bird eggs and the patterns that adorn them vary widely among species. Most non-passerines—including owls (Strigiformes), some ducks (Anatidae), pigeons (Columbidae), hummingbirds (Trochilidae) and woodpeckers (Picidae)—lay pure white eggs, without obvious maculation [1]. Unmaculated eggs are believed to be the ancestral state for Aves [2], although the earliest birds may have been capable of colouring their eggs, as has been reported in some dinosaurs [3]. By contrast, most Passeriformes—perching songbirds—lay maculated eggs, typically with reddish markings concentrated as a ring on the blunt end [4,5]. Nearly all colour of bird eggshells is derived from only two

pigments: protoporphyrin IX, responsible for the red-brown coloration, and biliverdin, for the blue-green coloration [6]. Eggshell maculation is thought to serve numerous functions, including crypsis, thermoregulation and microbial defence. In passerines, it has been proposed that maculation may additionally aid as a defence mechanism against possible attack from avian brood parasites. For example, the eggs of tawny-flanked prinias (*Prinia subflava*) have a uniform background colour with coloured blotches and fine squiggles scrawled over the entire shell, which are difficult to replicate by parasitic cuckoo finches (*Anomalospiza imberbis*), allowing hosts to identify and reject foreign eggs [4]. In some species, the markings reinforce structural integrity in regions where the eggshell has thinned [1], while in others patterning may serve as a sexually selected signal through which a female can advertise the quality of both herself and her imminent nestlings [7,8, and references therein]. Most cavity-nesters lay white eggs as they are already well hidden from predators and hard to see, so colour would be very little use as signals, while ground-nesting shorebirds that lay their eggs in exposed locations tend to have strongly patterned eggs that blend in well with their nest environment, demonstrably increasing offspring survival [9]. The extent of eggshell variation across birds is remarkable, but a function of egg patterning that has received comparably less attention is the surface texture it creates.

Surface texture, or surface topography, refers to the three-dimensional features of a surface, defined by surface orientation and roughness [10]. Surface roughness relates to its waviness (height) and asperity; the deviation of a surface from its mean plane is characterized by variance of the height to form peaks and valleys [11]. Roughness influences surface functionality—not only surface properties such as hydrophobicity, optical and plasmonic behaviour, adhesion and friction, but also bulk properties, such as fracture toughness and fatigue resistance [12, and references therein]. There are numerous examples of smooth and rough surfaces in nature, each fulfilling a specific function. Surface roughness impacts the ability of water droplets to attach to the surface, and thus impacts bacteria adhesion and removal. For example, fruits or vegetables with rough surfaces (e.g. oranges, avocados and cantaloupes) are better protected from bacterial infection compared to fruits with smooth surfaces (e.g. apples) [13]. Sun beetles (*Pachmoda* spp.) can more easily grasp rougher substrate particles with their claw tip compared to smooth substrate particles, as rough surfaces generate a greater friction force [14]. Similarly, the glossy appearance of tinamou eggshells is produced by an extremely smooth cuticle causing light to reflect in the specular direction, whereas the matt appearance of chicken (*Gallus gallus*) eggs is produced by having a rough cuticle, causing light to be scattered in multiple directions [15]. Thus, it is apparent that the texture of the surface has implications for bacterial removal, visual signalling and light responses. How these patterns pan out across a broader spectrum of the avian phylogeny has yet to be determined.

Mroz *et al.* [16] noted that the maculated eggs of turkey (*Meleagris gallopavo*) have a different surface structure on the foreground colour versus background colour, and in turn, this base colour was similar to immaculate eggs laid by the same species [16]. The difference in roughness between foreground and background pigment has never been properly quantified in birds, and it is unknown whether differences in

surface textures of immaculate and maculate eggs are universal, or what may be determining this potential difference. Based on Mroz's findings, Attard *et al.* [17] proposed that eggshell roughness will be greater on the foreground—the colour maculated parts—than the background base pigmentation of species with maculated eggs. In the present study we set out to test this. We hypothesized that (1) foreground pigmentation would be rougher and more peaked than background pigmentation, (2) there would be no distinction between the background pigmentation of maculated eggs and the pigmentation of immaculate eggs, and (3) variation in the surface topography of the foreground and background pigmentation would be influenced by life-history traits associated with the nest environment (see table 1 for full definitions and hypotheses). To test these hypotheses, we compiled high-resolution three-dimensional scans of avian eggshells from 486 species to characterize their surface structure.

## 2. Material and methods

### 2.1. Egg sampling

Empty eggshells from 486 species (1838 eggs) were sampled at the Western Foundation of Vertebrate Zoology (WFVZ) at Camarillo, United States of America and Natural History Museum (NHM) at Tring, United Kingdom. Fragments (surface area approx. 1.5 cm<sup>2</sup>) were cut from the equatorial region of each egg using a micro-tool rotary saw with diamond-coated thin cutting wheel (Dremel 4000, Bosch Leinfelden, Germany). Eggshell fragments were gently cleaned with a cotton bud dipped in distilled water, then allowed to dry for at least 24 h. Eggshells coated with a thin, chalky layer of vaterite were not cleaned as vaterite crystals dissolve upon contact with water [35].

### 2.2. Interferometer

The surface topography of eggshell surfaces was obtained using a three-dimensional non-contacting optical profilometer (LeicaSCAN DCM3D, Leica Microsystems, Germany) connected to a white light interferometric microscope. For each eggshell fragment, a section along the surface was scanned at three non-overlapping locations at a focal depth of 100 µm (100 focal planes at 1 µm resolution) using a 20× objective magnification to give a measurement area of 636.61 × 477.25 µm<sup>2</sup> (pixel resolution = 768 × 576).

During scanning, maculation was recorded for each eggshell fragment as 0—if the fragment was immaculate; 1—for maculation present but with a clear, dominant background colour that was able to be separated from the foreground; and 2—for widespread maculation that covered most of the fragment that could not be separated from background [36]. For maculated (spotted, squiggly or blotched) eggshells, the foreground and background pigment was scanned separately, each in three different locations (total 6 scans per fragment). Scans were manually cropped in Scanning Probe Image Processor, SPIP version 4.4.3.0 (Image Metrology, Hørsholm, Denmark) if both foreground and background pigments were in the image so only the region of interest remained. Eggs that were too difficult to divide into foreground and background pigments (e.g. densely speckled eggshells) were excluded from the study. Cropped scans, and scans that did not require cropping (total 7013 scans), were then transferred to SPIP for processing and analysis. We used the plane correction tool to automatically correct plane distortions in the data using polynomial functions. In this case, a second-order polynomial was used as the slope on the data was linear. The mean z-value was then adjusted to 0. Scans which had elevation data for less than 40% of pixels were manually inspected

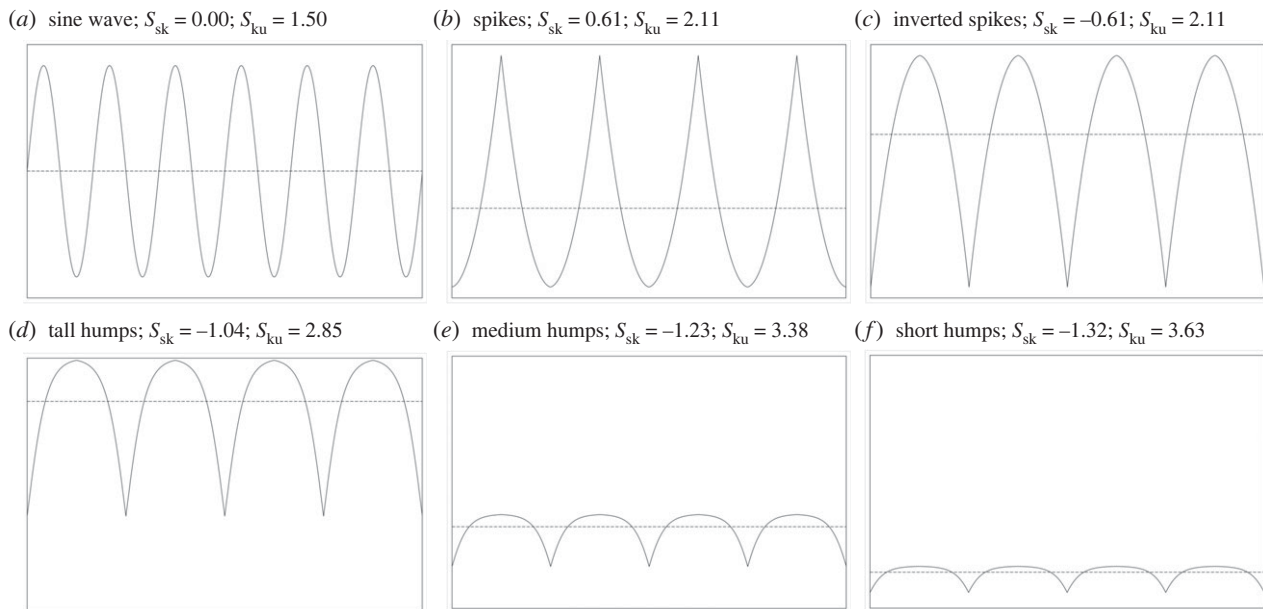
**Table 1.** Predictions and definitions for possible explanations for variation in eggshell topography in birds. Source lists references for definitions, and primary databases used to compile bird life-history traits. Hypotheses are numbered 1 to 13.

ID	predictor	logic	definition	source
1	body mass	as adult body mass is correlated with egg mass, eggshells of heavier birds will have rougher foreground and background pigment compared to eggshells of smaller birds	mean body mass (g) of adult birds	data from Dunning <i>et al.</i> [18], with updates from Wilman <i>et al.</i> [19] and Pigot <i>et al.</i> [20]. Database compiled by Sheard <i>et al.</i> [21]
2	clutch size	species with larger clutches will be more likely to experience greater friction from rubbing against adjacent eggs. Therefore, larger clutches will have rougher surfaces, particularly the foreground pigmentation	number of eggs per brood, measured as geometric mean of the typical minimum and maximum clutch size	databases from Jetz <i>et al.</i> [22], Lislevand <i>et al.</i> [23] and Myhrvold <i>et al.</i> [24]. Gaps filled in using HBW Alive [25] and other sources
3	diet	the composition of the eggshell is correlated with diet. Herbivores have thicker eggshells than invertivores and omnivores (M.R.G.A. and S.J.P., unpublished). As microbes can more easily enter the egg of thinner eggshells, species that consume insects or are omnivores will develop smoother, more hydrophobic eggshell surfaces to minimize microbial adhesion	plant: diet primarily consists of fruit, buds, seeds or plants insectivore: diet primarily consists of insects omnivore/carnivore: diet is omnivorous, carnivorous or a scavenger	category based on Wilman <i>et al.</i> [19], updated from HBW Alive [25] and other sources. Database from Sheard <i>et al.</i> [21]
4	mode of development	longer incubation duration will promote the accumulation of microbes on the eggshell surface. Precocial species require more incubation time than altricial species, thus are expected to possess smoother eggshell surfaces	altricial: newly born young are relatively immobile, naked, and usually require care and feeding by the parents precocial: newly born young are relatively mobile, covered in feathers, and independent	category based on Augustine <i>et al.</i> [26], Stark [27] and Stark & Ricklefs [28]. Data from HBW Alive [25] and other sources
5	nest type	nests in cavities or burrows have a higher relative humidity than open-top nests [29] and are more insulated [30]. As the level of bacterial penetration through the shell increases with higher temperature and relative humidity [31], the shell surfaces of eggs laid in enclosed nests will be rougher and more hydrophobic than eggs laid in semi-enclosed and exposed nests	exposed: nest is open above and has no side walls (no nest, scrape, saucer, platform, heap) semi-enclosed: nest is partially open and has side walls (cup, bowl, pendant, sphere, dome, pouch) enclosed: nest is entirely enclosed (cavity, burrow, crevice)	category from this paper. Data from HBW Alive [25] and other sources
6	nest location	elevated nests have lower risk of flooding, water accumulation or exposure to dirt and animal faeces, therefore will have smoother surfaces compared to burrows and ground-nesting species, due to reduced risk of infections	ground: nest location in or on the ground water: floating on water elevated: nest located in tree, bush, shrub, wall, cave roof, cliff or attached to reed	category based on Portugal <i>et al.</i> [32]. Data from HBW Alive [25] and other sources

(Continued.)

Table 1. (Continued.)

ID	predictor	logic	definition	source
7	habitat	eggs of species breeding in open habitats are more vulnerable to heat loss due to exposure to wind [33], therefore their eggshells are expected to have smoother surfaces to reduce heat loss compared to eggs of species breeding in semi-open and dense habitats	open: species primarily occurs in desert, grassland, open water, open moorland, low shrubs, rocky habitats, seashores and cities semi-open: species primarily occurs in open shrubland and bushland, scattered bushes, parkland, forest edge dense: species primarily occurs in forest with a closed canopy, or in the lower vegetation strata of dense thickets, shrubland, mangroves or marshland lined: nest lining is always or sometimes present not lined: nest lining is absent	habitat scores from Tobias <i>et al.</i> [34]. Database compiled by Sheard <i>et al.</i> [21]
8	nest lining	incorporation of nest lining will trap moisture, resulting in smoother surfaces	lined: nest lining is always or sometimes present not lined: nest lining is absent	category from this paper. Data from HBW Alive [25] and other sources
9	incubating parent	eggs are more prone to microbial penetration when the parent leaves the nest uncovered. This is more likely to occur if incubation is not shared between parents, hence these eggs are more likely to have smoother eggshells	not shared: contact incubation of eggs by single adult shared: contact incubation of eggs by two adults	category from Portugal <i>et al.</i> [32]. Data from HBW Alive [25] and other sources
10	parental contact	the wet incubating parent returning to the nest will increase the nest's humidity, thus eggs of these species are expected to have rougher eggshells	wet plumage: adults return habitually to the nest with wet plumage. This included species that feed on freshwater or marine prey, or use nests built on water dry plumage: adults did not return habitually to the nest with wet plumage	category from Portugal <i>et al.</i> [32]. Data from HBW Alive [25] and other sources
11	parental care	the eggshells of species that provide biparental care are expected to have rougher surfaces, as nest humidity and temperature can be better maintained when both parents assist	uniparental: the brood is provisioned and/or defended by one adult biparental: the brood is provisioned and/or defended by at least two adults	category from Portugal <i>et al.</i> [32]. Data from HBW Alive [25] and other sources
12	annual temperature	as the level of bacterial penetration through the shell increases with higher temperature [31], eggshells of eggs incubated in warmer climates will have smoother surfaces to avoid microbial colonization	average annual mean temperature (BI01) of breeding/ resident range	from Sheard <i>et al.</i> [21], based on WorldClim v1 data [21]
13	annual precipitation	eggshells incubated in environments with higher annual precipitation will be rougher, to combat temporary periods of excessive rain	average annual mean precipitation (BI012) of breeding/ resident range	from Sheard <i>et al.</i> [21], based on WorldClim v1 data [21]



**Figure 1.** Schematic visualizing relative values of  $S_{sk}$  and  $S_{ku}$ . (a) Sine wave;  $S_{sk} = 0.00$ ;  $S_{ku} = 1.50$ ; immaculate example: ruby-throated hummingbirds, *Archilochus colubris* ( $S_{sk} = -0.02$ ,  $S_{ku} = 2.80$ ). (b) Spikes;  $S_{sk} = 0.61$ ;  $S_{ku} = 2.11$ ; immaculate examples: wood storks, *Mycteria americana* ( $S_{sk} = 0.56$ ,  $S_{ku} = 3.59$ ) or black-necked stilts, *Himantopus mexicanus* ( $S_{sk} = 0.62$ ,  $S_{ku} = 3.92$ ). (c) Inverted spikes;  $S_{sk} = -0.61$ ;  $S_{ku} = 2.11$ ; immaculate example: barred cuckoo-doves, *Macropygia unchall* ( $S_{sk} = -0.61$ ,  $S_{ku} = 3.27$ ) or red-shouldered hawks, *Buteo lineatus* ( $S_{sk} = -0.60$ ,  $S_{ku} = 3.62$ ). (d) Tall humps;  $S_{sk} = -1.04$ ;  $S_{ku} = 2.85$ ; immaculate example: brown pelicans, *Pelecanus occidentalis* ( $S_{sk} = -1.02$ ,  $S_{ku} = 4.87$ ). (e) Medium humps;  $S_{sk} = -1.23$ ;  $S_{ku} = 3.38$ ; immaculate example: firewood-gatherers, *Anumbius annumbi* ( $S_{sk} = -1.08$ ,  $S_{ku} = 6.16$ ). (f) Short humps;  $S_{sk} = -1.32$ ;  $S_{ku} = 3.63$ ; immaculate example: Manx shearwaters, *Puffinus puffinus* ( $S_{sk} = -1.48$ ,  $S_{ku} = 7.09$ ). All of the values are dimensionless. The dashed line indicates the average height of the line section. All figures are generated from equations and are not the actual species examples mentioned.

in SPIP to determine if the scan quality was sufficient for analysis. Some scans were cropped to remove low quality regions of the scan for analysis. Scans were taken under a narrow focal plane, so the centre of the field of view usually captured the topography well and were suitable for inclusion in the analysis, even where pixel information was absent around the scan edges. Scans of brood parasite eggshells (total 40 scans from 5 brood parasite species) were excluded from the study, as they represent a unique example of an unusual breeding strategy, which benefits from specific eggshell adaptations [37]. However, we have provided eggshell surface texture values for brood parasites that were scanned within the Figshare repository.

After removing brood parasites, surface texture values from multiple locations on the same egg were averaged for immaculate eggs, and maculated eggs with a score of 1 to obtain a specimen mean value. Cook's distance was applied to specimen  $S_{ar}$ ,  $S_{sk}$  and  $S_{ku}$  values (see below for definitions) using R statistical software (R v4.1.2) [38] to identify outliers and/or influential values, which were subsequently removed from analysis [39]. We retained surface texture measurements from 1873 specimens across 345 species after removing low-quality scans and influential values.  $S_{ar}$ ,  $S_{sk}$  and  $S_{ku}$  values were averaged per species from specimen-specific values for phylogenetic comparative analyses.

Scans were processed using SPIP to quantify surface roughness ( $S_{ar}$ , nm), surface skewness ( $S_{sk}$ ) and surface kurtosis ( $S_{ku}$ ) (figure 1).  $S_a$  expresses, as an absolute value, the difference in height of each point compared to the arithmetical mean of the surface [40]. Surface skewness describes the asymmetry of the height distribution histogram. If  $S_{sk} = 0$ , a symmetric height distribution is indicated. If  $S_{sk} < 0$ , it can be a bearing surface with holes and if  $S_{sk} > 0$  it can be a flat surface with peaks.  $S_{sk}$  values numerically greater than 1.0 may indicate extreme holes or peaks on the surface [40]. The surface kurtosis ( $S_{ku}$ ) describes the 'peakedness' of the surface topography. Smaller values indicate broader height distributions and vice versa for values greater than 3.0.  $S_{ar}$ ,  $S_{sk}$  and  $S_{ku}$  are based on surface height distribution [41] and are scale-dependent (figure 1). Therefore, these measures depend

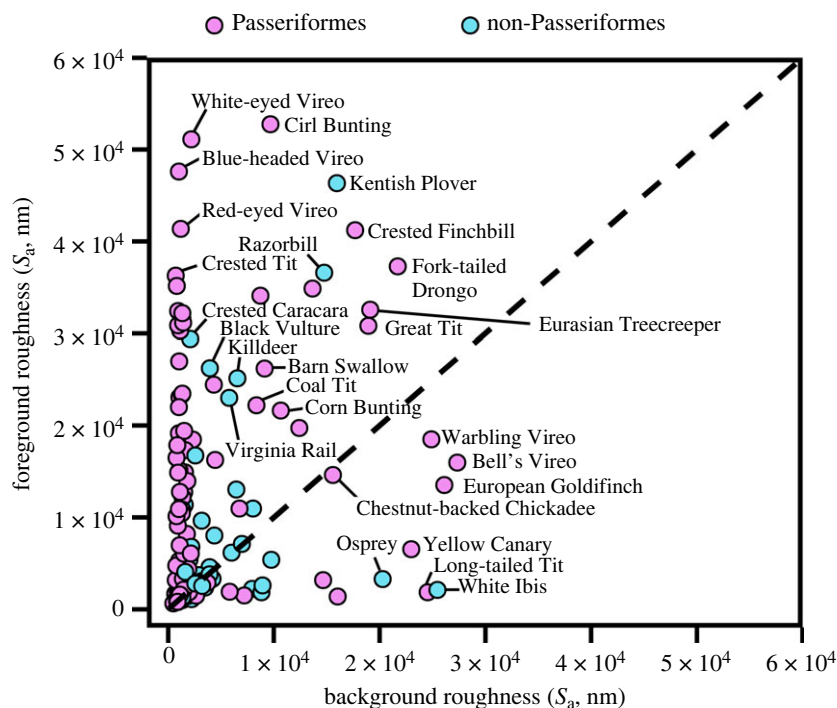
on the available measurement scale and the sampling interval of the measurement technique. As the distributions of  $S_a$  and  $S_{ku}$  values across species were skewed, we log-10 transformed these response variables to achieve a normal distribution for statistical analysis.  $S_{sk}$  values were not transformed as they had a normal distribution across species.

### 2.3. Life-history and ecological data

We collected literature data on 13 life-history traits (table 1) that represent different ecological and mechanistic causes of eggshell surface texture heterogeneity across modern birds. Ecological explanations address evolutionary function, such as why eggshell heterogeneity in maculated eggs exists, while mechanistic explanations address how interspecific differences in eggshell texture are achieved [39]. Table 1 lists the hypotheses, rationale and definitions of each predictor. The life-history traits investigated include different aspects of parent behaviour, embryo development, nest environment and climate conditions that could potentially influence the evolution of avian eggshell texture, and surface heterogeneity in maculated eggs.

### 2.4. Phylogenetic comparative analysis

Phylogenetic comparative analyses and plots were processed in R. To analyse the evolution of surface texture among maculate and immaculate bird eggshells within a comparative context, we used the most complete molecular phylogeny of extant bird species (available from <http://www.birdtree.org>) to generate 10 000 trees using the primary backbone of Hackell *et al.* [42] for all species in our study. Pagel's lambda ( $\lambda$ ) was used to determine the extent of phylogenetic signal for  $S_{ar}$ ,  $S_{sk}$  and  $S_{ku}$  for the foreground and background pigment of maculated eggs and eggshell surface of immaculate eggs. Phylogenetic signal was measured using the *phylosig* function in the package 'phytools' [43]. At  $\lambda = 0$  the trait of interest may vary randomly across a



**Figure 2.** Eggshell surface roughness ( $S_a$ , nm) of foreground and background pigment of 204 bird species with maculated eggs. Dotted line has a slope of 1, with mean species  $S_a$  values above the line having rougher foreground versus background pigment surface. Species are colour coded based on avian clade (pink = Passeriformes; blue = non-Passeriformes) and a subset of species are labelled. The data in the figure are not corrected for phylogenetic relatedness.

phylogeny while at  $\lambda = 1$ , closely related species tend to exhibit more similarity in trait expression.

The association between eggshell texture and life-history traits was assessed using phylogenetic generalized least squares (*pgls*) regression models to account for phylogenetic uncertainty in comparative analyses across species. Each *pgls* model tested one or more comparative hypotheses using an entire set of equally likely trees. We ran two simple *pgls* models: firstly, to test if the foreground to background pigmentation of maculated (patterned) eggs is significantly different, and secondly, to test if the base colour of species with maculated eggs is significantly different from that of species with immaculate (non-patterned) eggs.

For each maculated egg, we calculated the difference between foreground and background pigment  $S_a$ ,  $S_{sk}$  and  $S_{ku}$  values to assess the degree of contrast in surface texture, referred to herein as  $\Delta S_a$ ,  $\Delta S_{sk}$  and  $\Delta S_{ku}$ , respectively. More positive values indicated the foreground pigment has greater roughness or texture than the background pigment. This ratio was averaged across all eggs from the same species to use as the response variable for *pgls* modelling to investigate drivers of species-specific differences in the contrast ratio between foreground and background surface textures across species that lay maculated eggs. We ran a separate series of *pgls* models using actual values of foreground and background pigmentation for maculated egg as the response variable and included climate and life-history traits as predictors. Likewise, we ran a separate series of *pgls* models to assess the influence of life-history traits on the evolution of eggshell surface topography among species that lay immaculate eggs. Sample sizes and categorical predictors included in each *pgls* analysis are provided in electronic supplementary material, table S1. Pagel's lambda ( $\lambda$ ) [44] was measured for foreground, background and delta values using the package 'phytools' [45] to determine to what extent related species were more likely to share similar roughness values for maculated eggs.

We tested for collinearity among pertinent life-history traits, and only selected uncorrelated variables (with paired-correlation

less than 0.75) and VIF under 10 as predictor variables (see Attard *et al.* [17,39] for details). We included up to 5 predictors per *pgls* model. For *pgls* using multiple predictors, we performed conditional model-averaging for *pgls* models with a delta Akaike information criterion (AICc) score of  $\leq 2$  relative to the top-ranked model. Model averaging allows us to average the parameter estimates across multiple models that best explain the data and avoids the issue of model uncertainty [46].

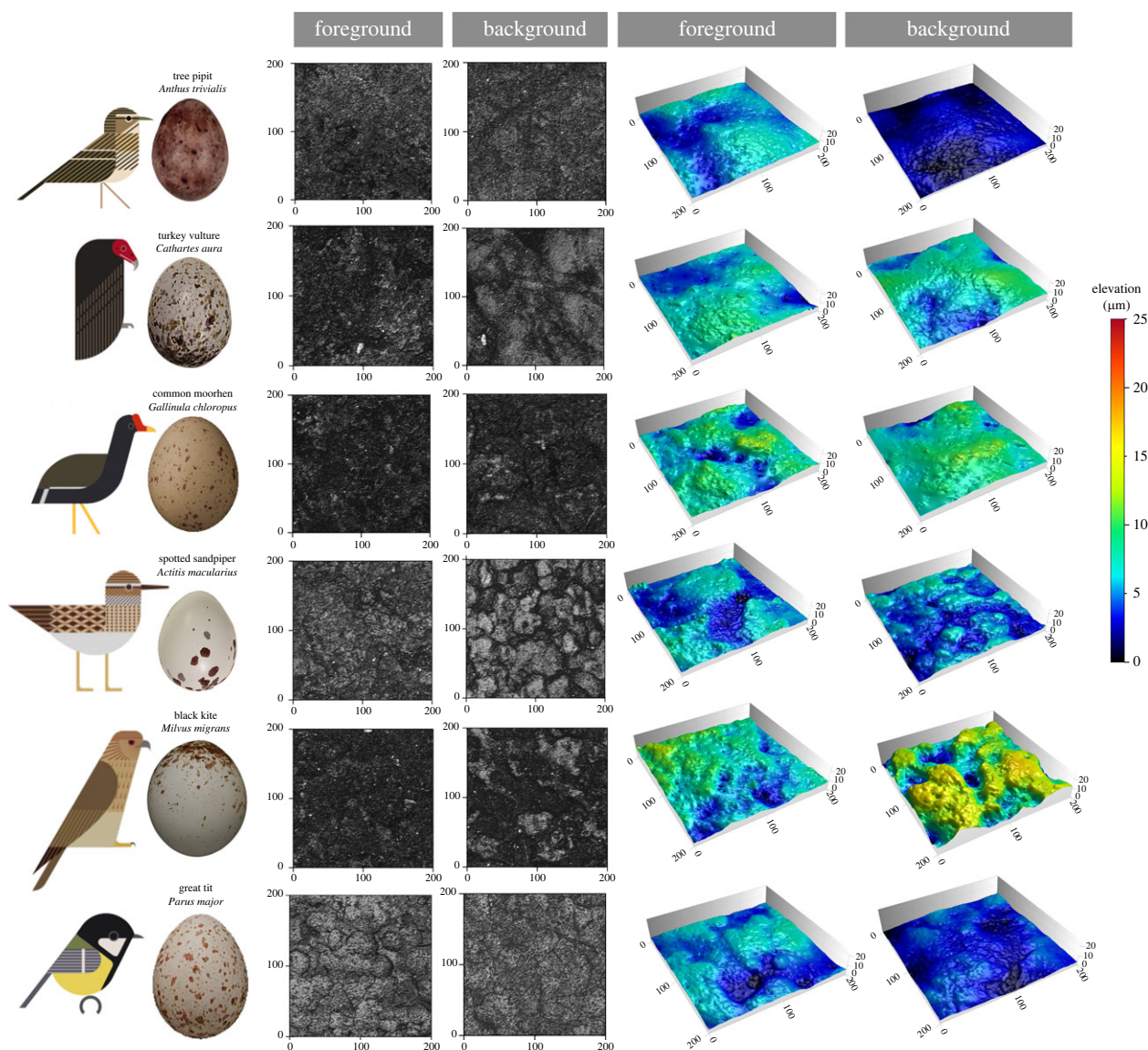
### 3. Results

In this study, we measured and compared the surface roughness ( $S_a$ ), surface skewness ( $S_{sk}$ ) and surface kurtosis ( $S_{ku}$ ) among maculated and immaculate avian eggshells. These parameters describe aspects of surface texture.  $S_a$  is defined as the difference in height of each point compared to the surface average,  $S_{sk}$  is the degree of asymmetry of the height distribution histogram (i.e. if the surface is flat with peaks or is a surface with holes), and  $S_{ku}$  describes the broadness of the surface peaks.

#### 3.1. Differences in pigment surface topography in patterned and non-patterned avian eggshells

The correlations between  $S_a$ ,  $S_{ku}$  and  $S_{sk}$  for foreground pigment, background pigment and pigmentation on immaculate eggs were weak, confirming that these surface structure variables provide different quantitative information about eggshell surface characteristics (electronic supplementary material, table S2). As such, each of these values were used to represent different aspects of eggshell surface texture in our analysis.

Among the 187 species analysed with maculated eggs, 71% had higher  $S_a$  values for the foreground than background regions (figure 2). Digital elevation models for several species with the most extreme difference in foreground



**Figure 3.** Surface topography of maculated eggshells from a selection of species included in this study. Greyscale two-dimensional images of the surface topography are shown for the foreground and background pigment. Digital elevation models of the foreground and background pigment for one specimen per species ( $1\ \mu\text{m}$  resolution, dimensions  $200\ \mu\text{m} \times 200\ \mu\text{m}$ ). Bird art by Scott Partridge and egg photos by the Western Foundation of Vertebrate Zoology. The egg photos are not to scale. More information can be found in the electronic supplementary material.

and background roughness are shown in figure 3.  $S_{sk}$  and  $S_{ku}$  values were higher in the foreground versus background pigmentation for 55% and 47% of species, respectively. There was a significant positive correlation in the foreground region relative to the background region for  $S_a$  ( $pgls$ : Estimate = 0.43,  $R^2 = 0.10$ ,  $p < 0.001$ ) and  $S_{sk}$  ( $pgls$ : Estimate = 0.24,  $R^2 = 0.05$ ,  $p < 0.001$ ), meaning that when foreground pigment has higher eggshell roughness and symmetry of surface heights above the mean plane, so too will the background pigment. However, there was no association between foreground and background  $S_{sk}$  ( $pgls$ : Estimate = 0.02,  $R^2 < 0.01$ ,  $p = 0.70$ ) values. Together, this suggests that overall, among maculated eggs, the foreground pigment tends to be rougher and slightly more symmetrical for any peaks present compared to background pigment, but differences in surface peakedness is variable between species. Species that lay immaculate eggs ( $n = 174$ ) showed no difference in  $S_a$  ( $R^2 = -0.00$ ,  $p = 0.34$ ),  $S_{ku}$  ( $R^2 = -0.05$ ,  $p = 0.99$ ) and  $S_{sk}$  ( $R^2 = -0.02$ ,  $p = 0.43$ ) values compared to the background pigment of species with maculated eggs ( $n = 192$ ).

### 3.2. Phylogenetic signal for eggshell surface topography

There was a weak phylogenetic signal for surface texture difference between foreground and background pigment ( $\lambda = 0.06$  for  $\Delta S_a$ , and  $\lambda = 0.00$  for  $\Delta S_{sk}$  and  $\Delta S_{ku}$ ), which were significantly different from 1 ( $p < 0.001$ ), but not significantly different from 0 ( $p = 0.22$ – $1.00$ ) (table 2). This means that heterogeneity in eggshell surface texture among species with maculated eggs has evolved mainly independently of phylogeny and close relatives are no more similar than distant relatives [47]. The phylogenetic signal for most eggshell topographical measures was low ( $\lambda = 0.00$ – $0.39$ ) and significantly different from 1 ( $p < 0.001$ ) for maculated eggs when pigment types were analysed separately. For foreground and background colour, the phylogenetic signal for  $S_{sk}$  was significantly different from 0 and 1 ( $p < 0.001$  and  $p < 0.001$ , respectively), whereas  $S_a$  and  $S_{ku}$  were not significantly different from 0 (table 2). Species with immaculate eggs had no phylogenetic signal for  $S_{sk}$  ( $\lambda = 0.00$ ,  $p = 1.00$  for  $\lambda = 0$ ,  $p < 0.001$  for  $\lambda = 1$ ), and intermediate phylogenetic signal for  $S_a$  ( $\lambda = 0.30$ ,  $p = 0.03$  for  $\lambda = 0$ ,  $p < 0.001$  for  $\lambda = 1$ )

**Table 2.** Estimates of phylogenetic signal in surface roughness ( $S_a$ ), surface skewness ( $S_{sk}$ ) and surface kurtosis ( $S_{ku}$ ) in all birds, separated based on pigmentation as follows: (1) difference in surface texture between foreground to background pigmentation of maculated eggs, (2) surface texture of foreground and background of maculate eggs analysed separately, and (3) surface texture of immaculate eggs.  $S_a$  and  $S_{ku}$  were log-10 transformed prior to analysis. The  $p$ -value tests the null hypothesis for both no phylogenetic signal ( $\lambda = 0$ ) and a Brownian motion model ( $\lambda = 1$ ) of evolution, and are shown in parentheses under the log-likelihood ratios.

egg type	pigment	response variable	Pagel's $\lambda$	log likelihood	log likelihood for model $\lambda = 0$	log likelihood for model $\lambda = 1$
maculated	difference between foreground and background	$\Delta S_a$	0.06	-2036.95	1.47 (0.22)	-224.97 (<0.001)
		$\Delta S_{sk}$	0.00	-53.78	-0.003 (1.00)	-104.99 (<0.001)
		$\Delta S_{ku}$	0.00	-373.84	-0.01 (1.00)	-434.58 (<0.001)
	foreground	$S_a$	0.39	-150.98	3.49 (0.06)	-228.57 (<0.001)
		$S_{sk}$	0.10	-14.92	8.59 (<0.01)	-98.60 (<0.001)
		$S_{ku}$	0.13	190.04	-6.77 (0.01)	-91.92 (<0.001)
	background	$S_a$	0.07	-116.50	1.44 (0.23)	-196.84 (<0.001)
		$S_{sk}$	0.20	-19.46	18.77 (<0.001)	-101.10 (<0.001)
		$S_{ku}$	0.06	149.46	1.84 (0.17)	38.63 (<0.001)
immaculate	immaculate eggs	$S_a$	0.30	-121.78	4.73 (0.03)	-196.01 (<0.001)
		$S_{sk}$	0.00	-70.70	-0.00 (1.00)	-117.33 (<0.001)
		$S_{ku}$	0.68	74.30	16.45 (<0.001)	-23.08 (<0.001)

and  $S_{ku}$  ( $\lambda = 0.68$ ,  $p < 0.001$  for  $\lambda = 0$  and  $\lambda = 1$ ). Among species that lay immaculate eggs,  $S_{sk}$  has evolved independently of phylogeny, whereas both phylogeny and life-history contribute to the evolution of  $S_a$  and  $S_{ku}$ .

### 3.3. Association between topographic heterogeneity and life-history among maculate eggshells

Species that occupy dense habitats had higher  $\Delta S_a$  than those in semi-open ( $z = 2.01$ ,  $p = 0.04$ ) and open habitats ( $z = 2.29$ ,  $p = 0.01$ ) (figure 4; electronic supplementary material, table S3). Dense habitats included forests with a closed canopy, or species that live in lower vegetation strata of dense thickets, shrubland, mangroves or marshland, whereas open habitats included deserts, open water, grassland, seashores and cities, and semi-open habitats included open shrubland, parkland and forest edges [34]. None of the life-history traits were significantly associated with  $\Delta S_{sk}$  or  $\Delta S_{ku}$  (electronic supplementary material, tables S4 and S5).

### 3.4. Influence of life-history on foreground and background eggshell pigmentation

When running *p*gls models for  $S_a$ ,  $S_{sk}$  and  $S_{ku}$  against body mass, background  $S_a$  and foreground  $S_{ku}$  values were significantly (positively) associated with body mass (Estimate = 0.10,  $R^2 = 0.02$ ,  $p = 0.03$  and Estimate = 0.03,  $R^2 = 0.06$ ,  $p < 0.001$ , respectively). Background  $S_{ku}$  significantly increased with higher annual precipitation ( $z = 2.72$ ,  $p < 0.01$ ; figure 5a) and clutch size ( $z = 0.05$ ,  $p = 0.02$ ; figure 5b) (electronic supplementary material, table S6). Background  $S_{sk}$  was negatively associated with larger clutches ( $z = 3.70$ ,  $p < 0.001$ ; figure 5c) and higher annual temperature ( $z = 2.23$ ,  $p = 0.03$ ; figure 5d) (electronic supplementary material, table S7). Species with altricial young had lower  $S_{sk}$  background values than species with precocial young ( $z = 4.84$ ,  $p < 0.001$ ; figure 5e). None of

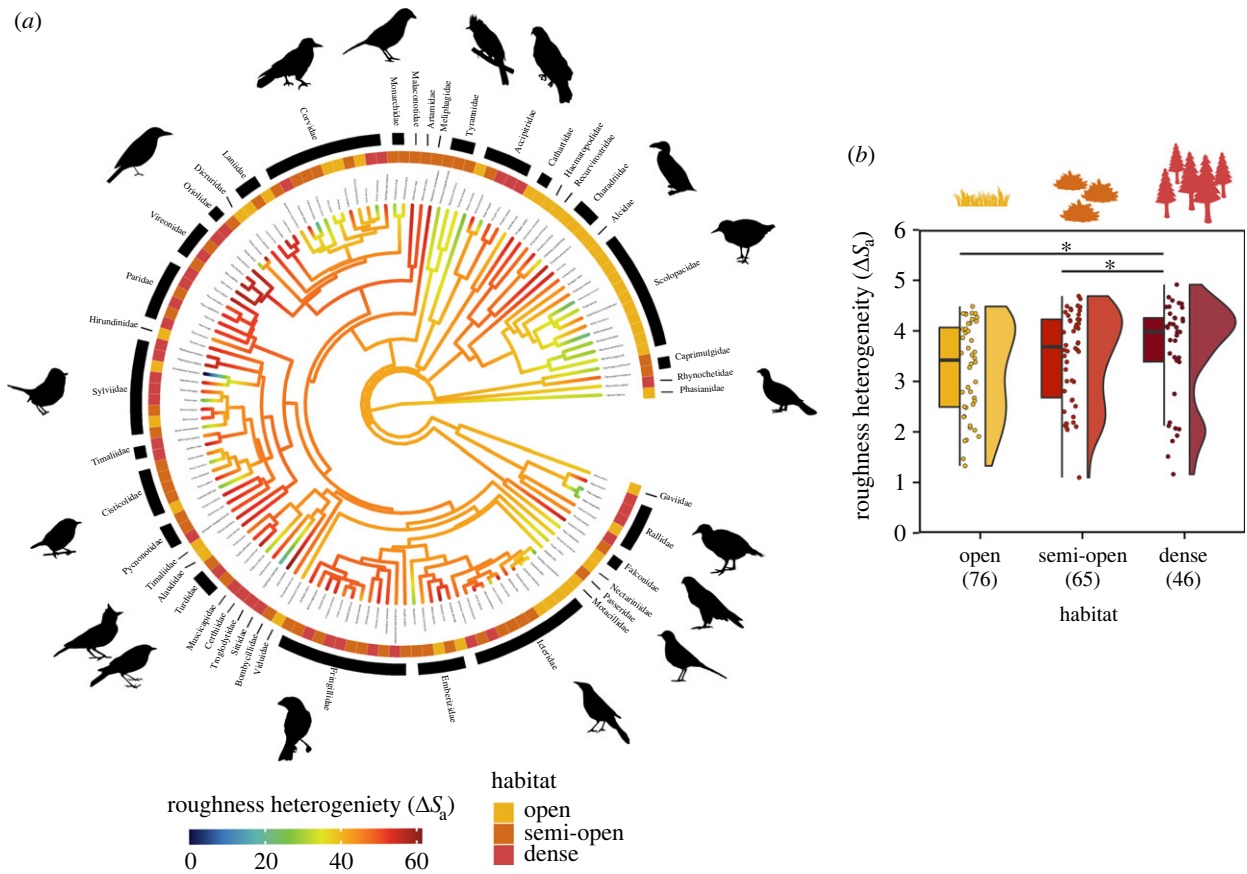
the life-history traits were significantly associated with  $S_a$  for the background pigment (electronic supplementary material, tables S8 and S9).

$S_a$  of the foreground pigment was lower among species occupying open habitats compared to dense habitats ( $z = 3.20$ ,  $p = 0.001$ ; figure 6a), species that return to the nest with wet plumage ( $z = 1.99$ ,  $p = 0.05$ ; figure 6b), and omnivores compared to herbivores ( $z = 2.02$ ,  $p = 0.04$ ; figure 6c) and insectivores ( $z = 2.93$ ,  $p = 0.001$ ; figure 6c) (electronic supplementary material, table S09). Species that nest close to (or on) water had higher foreground  $S_{ku}$  than species that nest above ( $z = 2.98$ ,  $p < 0.01$ ) or on the ground ( $z = 3.02$ ,  $p < 0.01$ ) (figure 6d; electronic supplementary material, table S11). Foreground  $S_{sk}$  was significantly higher in species that nest on the ground than above ground ( $z = 2.59$ ,  $p = 0.01$ ; figure 6e), and in Passeriformes compared to non-Passeriformes ( $z = 2.59$ ,  $p = 0.01$ , figure 6f). Species that lay their eggs in enclosed nests also had higher foreground  $S_{sk}$  compared to species that use exposed ( $z = 2.77$ ,  $p = 0.01$ ) or semi-enclosed ( $z = 2.02$ ,  $p = 0.04$ ) nests (figure 6g; electronic supplementary material, table S11).

### 3.5. Association between eggshell surface texture and life-history among immaculate eggshells

Among the 174 species analysed with immaculate eggs,  $S_a$  was found to be significantly higher in herbivores than omnivores ( $z = 3.68$ ,  $p < 0.001$ ), insectivores ( $z = 3.12$ ,  $p < 0.001$ ) and carnivores ( $z = 1.98$ ,  $p = 0.05$ ) (figure 7a), and was negatively associated with clutch size ( $z = 2.60$ ,  $p = 0.01$ ; figure 7b) (electronic supplementary material, table S12). None of the life-history traits were significantly associated with  $S_{ku}$  or  $S_{sk}$  among species with immaculate eggs, based on conditionally averaged models (electronic supplementary material, tables S13 and S14).





**Figure 4.** (a) Phylogenetic tree showing significant predictors of surface heterogeneity ( $\Delta S_a$ ) among maculated eggs. Branch colours depicts  $\Delta S_a$  (nm) for each species. Scientific names are shown as tip labels and avian family is separated using black bars. (b) Boxplot and violin plot showing the difference in foreground to background roughness as a function of habitat. Species that occupy dense habitats had higher  $\Delta S_a$  than those in semi-open and open habitats. A single asterisk (\*) signifies significant ( $p < 0.05$ ) differences for pairwise comparisons. Panel (b) is not corrected for phylogenetic relatedness. Silhouette illustrations came from PhyloPic (<http://phylopic.org>) and SVG Silh (<https://svgsilh.com/>), contributed by various authors under public domain licence (see electronic supplementary material).

## 4. Discussion

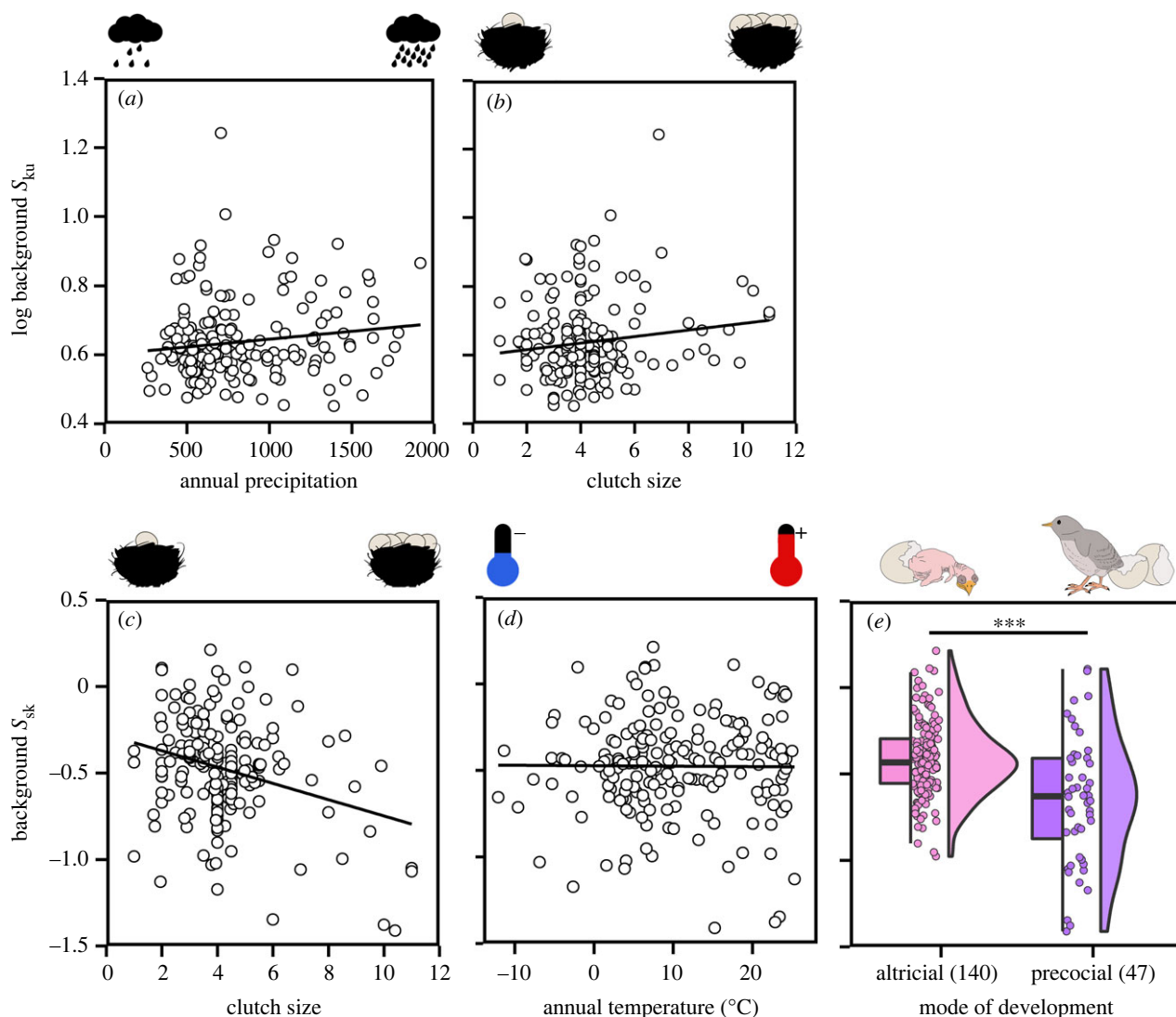
We found that the surface of maculated eggs consists of a rougher foreground pigment compared to the background pigment across 71% of the 204 bird species (54 families) investigated. For maculated eggs, the texture of the foreground was primarily correlated with parental care, habitat, diet, nest location and nest type, while background texture was correlated with annual temperature, clutch size, development mode and annual precipitation. For those species which lay immaculate eggs, there were no differences in surface roughness ( $S_a$ ), kurtosis ( $S_{sk}$ ) or skewness ( $S_{ku}$ ) compared to the background regions of maculated eggs. Surface roughness among immaculate eggs was greatest for herbivores, and species that have larger clutch sizes. The phylogenetic signal was low for our measured traits, suggesting that multiple life-history traits are likely governing the evolution of eggshell surface textures, and the eggshell surface properties measured have evolved independently of phylogeny; close relatives are no more similar than distant relatives.

### 4.1. Surface roughness ( $S_a$ )

The phylogenetic signal for  $S_a$  was low, suggesting life-history traits are contributing more to the relationships between background and foreground  $S_a$ . Despite this, none of our measured life-history traits were highlighted as significantly influencing  $S_a$ , potentially meaning an alternative trait not included in our analyses may be determining  $S_a$ . One

such factor may be dominant nest material; we did not include nest materials in our analyses due to the high variability of such materials used within a species. Another element it was not possible to determine for heavily maculated eggs was the percentage of surface area covered with foreground versus background pigment. Thus, it is feasible that an eggshell could exhibit a high foreground-to-background ratio, but the functionality of that ratio may be unclear if the foreground pigment consists of only a few spots in total.

Among the non-Passeriformes, foreground  $S_a$  was highest for Kentish plovers (*Charadrius alexandrinus*), razorbills (*Alca torda*), black vultures (*Coragyps atratus*) and killdeers (*Charadrius vociferus*). Killdeer and Kentish plovers are ground-nesting shorebirds, and their nests are a shallow depression or scape lined with shells, pebbles, grass and leaves [48,49]. Patterning on plover eggs has been proposed to help strengthen the eggshells along with providing crypsis [50,51]. Killdeer prefer lighter-coloured nesting materials, either to help keep the nest cool or to conceal it [48]. In Kentish plover eggshells, the relative quantities of protoporphyrin to biliverdin are positively correlated with the fractional dimension (a higher fractional dimension correlates with greater surface complexity) of spottiness, meaning that a combination of eggshell patterning characteristics (spot size and distribution, degree of spottiness and convolution of the spot outlines) influence the relative quantities of each pigment. Eggshell patterning is proposed to partly determine the propagation of cracks [52], as eggshells with a higher fractional



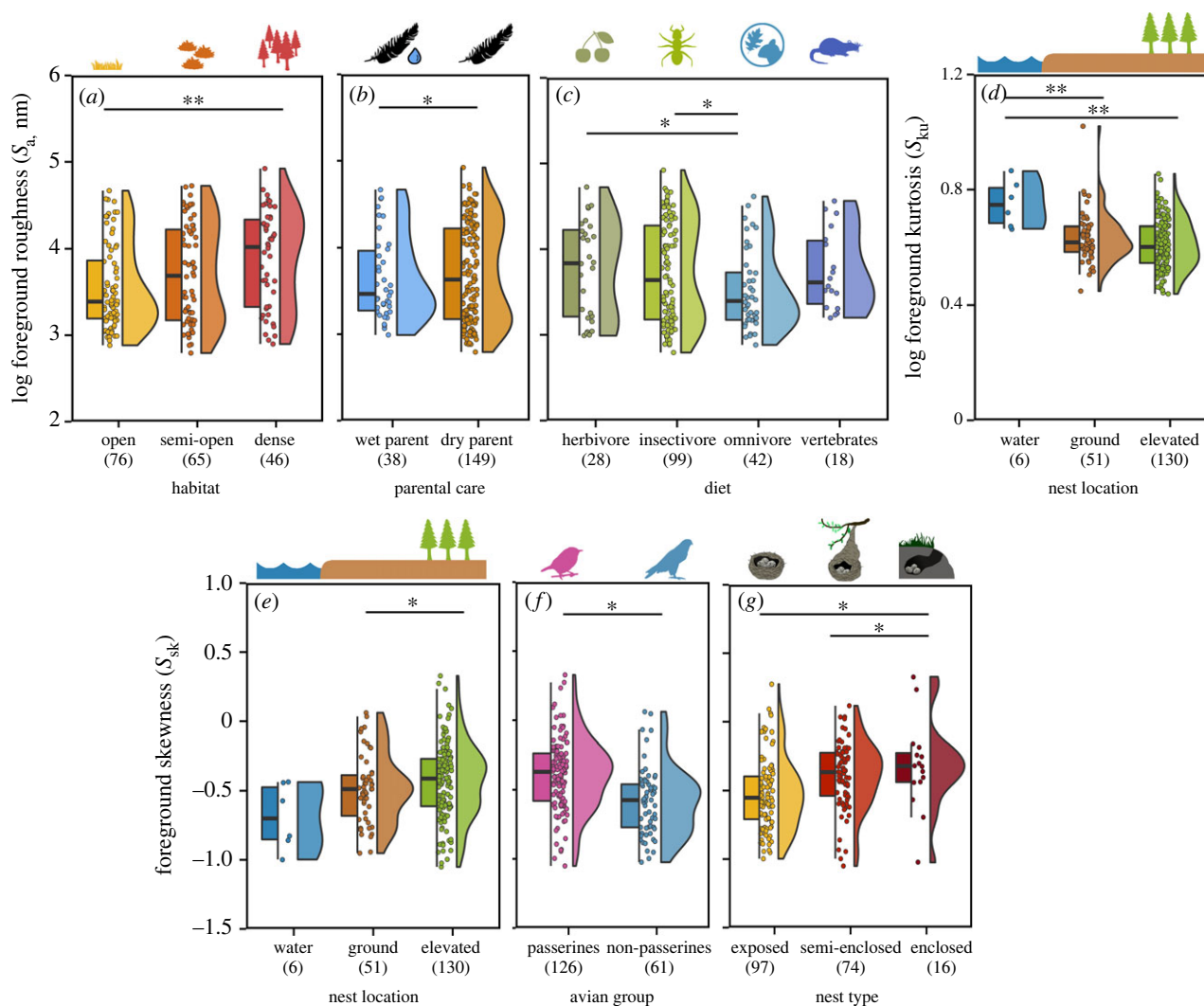
**Figure 5.** Significant predictors of eggshell surface kurtosis ( $S_{ku}$ ) and skewness ( $S_{sk}$ ) for background pigment among species that lay maculated eggs. Background  $S_{ku}$  is plotted as a function of (a) annual precipitation and (c) clutch size. Background  $S_{sk}$  is plotted as a function of (b) annual temperature, (d) clutch size and (e) mode of development. Three asterisks (\*\*\*) signify  $p < 0.001$  for pairwise comparisons. The data in the figures are not corrected for phylogenetic relatedness. None of the life-history traits were significant for background surface roughness ( $S_a$ ) in conditionally averaged models. Silhouette illustrations came from SVG Silh (<https://svgsilh.com>) under public domain licence (see electronic supplementary material).

dimension of spottiness may require more fracture energy to crack than maculated eggs with a lower fractional dimension, and therefore should be more resistant to breakage [50]. In our study, we found that most species with maculated eggs had a rougher foreground pigment compared to the background pigment. As crack length in heterogeneous materials is smaller than in more homogeneous materials [50], we propose that eggs of species with a greater difference in surface texture among foreground and background pigments are more resistant to crack propagation.

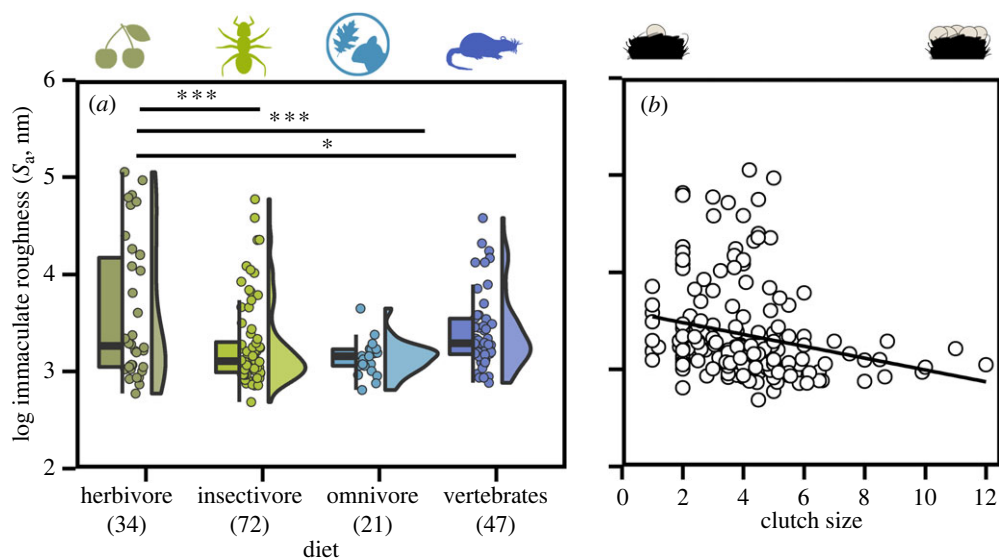
#### 4.2. Surface skewness ( $S_{sk}$ )

We found that species which nest on the ground, have enclosed nests, or are passerines have a higher foreground  $S_{sk}$  compared to the background part of the shell. A high skewness reflects more 'pointed' peaks with a flatter surface between peaks, while low skewness describes more 'rounded' peaks and no flat surface between peaks. Thus, such holes in the background component of the eggshell may indicate the presence of pores, suggesting pores on the eggshell surface of ground-nesting species, those in enclosed nests and those in the passerines

group are present more in the background of the shell, rather than the foreground pigmented regions. In certain bird species studied (e.g. *Gavidae*) it has been observed that pigment can run down the pore channel and into the mammillary cones at the bottom [53], suggesting that pores are not exclusively found only in the background part of the shell, but also found in what would be considered the foreground pigmented areas. In many instances, pore mouths are not visible on the surface at all, often instead being hidden from view by the cuticle [53]. However, there can be indications of pore openings present in the deeper valley-like regions of the shell, and in those species where a cuticle is entirely absent [54]. The distribution and difference in pore openings between species, and whether these coincide with foreground pigment spotting, could potentially be linked to the presence or absence of a cuticle, or to specific structures that are found on eggs that potentially deal with heavy dirt or detritus. For example, eggshell cuticles—made of organic matter—are prevalent in precocial species (e.g. common quails, common ostriches (*Struthio camelus*), greylag geese), but absent in many altricial species studied (e.g. feral pigeons (*Columba livia*), canaries (*Serinus canaria*), budgerigars (*Melopsittacus undulatus*)) [54].



**Figure 6.** Significant predictors of foreground surface roughness ( $S_a$ , nm), kurtosis ( $S_{ku}$ ) and skewness ( $S_{sk}$ ) among species that lay maculated eggs. Foreground  $S_a$  is plotted as a function of (a) habitat, (b) parental contact, and (c) diet. Foreground  $S_{ku}$  is plotted as a function of (d) nest location, while foreground  $S_{sk}$  is plotted as a function of (e) nest location, (f) avian group and (g) nest type. The data in the figures are not corrected for phylogenetic relatedness. Significant differences between categorical variables based on conditionally averaged models are given in asterisks with \*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$ . Silhouette illustrations came from PhyloPic (<http://phylopic.org>) and SVG Silh (<https://svgsilh.com/>), contributed by various authors under public domain licence (see electronic supplementary material).



**Figure 7.** Significant predictors of surface texture among species that lay immaculate eggs. Surface roughness ( $S_a$ ) is plotted as a function of (a) diet and (b) clutch size. Significant differences between categorical variables based on conditionally averaged models are given in asterisks with \*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$ . Silhouette illustrations came from the authors or Silh (<https://svgsilh.com/>) under public domain licence (see electronic supplementary material).

A lack of cuticle may explain why we found that  $S_{sk}$  in the background colour of the eggshell of altricial species was lower when compared to those which are precocial. A total lack of a cuticle will have implications for the overall eggshell surface heterogeneity and topography, particularly influencing how pores appear on the outer surface of the shell, and thus influencing  $S_{sk}$ .

Pore structure itself may further influence the degree of peakedness of the surface ( $S_{ku}$ ) and overall surface topography. Although pores are typically—but not always—covered in amorphous organic matter, different pore types may influence the  $S_{ku}$  of the egg surface. For example, pore types that are characterized by extensive branched pores venting into grooves present in the external shell surface (seen in, e.g. American rheas (*Rhea americana*)) [55] are likely to have a greater influence on  $S_{ku}$  and  $S_{sk}$  than those pore types such as a simple, unbranched, funnel-shaped pore, seen in many bird species [55]. For certain species, particularly those typically associated with aquatic environments, unique eggshell structures are present, which in turn are likely to influence  $S_a$ ,  $S_{sk}$  and  $S_{ku}$ . For example, spheres on the outer surface of the shell of grebes (Podicipediformes) contain calcium phosphate [56], while the shells of cormorants (Phalacrocoracidae), flamingos (Phoenicopteridae) and anis (Crotophaga) contain the calcium polymorph vaterite [35,57,58]. The implications of these structures on overall surface heterogeneity is currently poorly understood.

For a number of species, further examination of the brown (likely protoporphyrin) pigment spots showed the presence of needle-shaped calcite crystals which were morphologically markedly different from the main calcite crystals [50]. This morphological difference in pigment calcite crystals has not been quantified across a variety of species, but may be a contributing factor to both the difference in our measured parameters ( $S_a$ ,  $S_{sk}$ ,  $S_{ku}$ ) between species, and also between the background and foreground measurements. Although a focus of our study was the comparison of background and foreground parts of the eggshell, immaculate eggshells are not themselves devoid of pigment. The eggshells of scops owls (*Otus scops*), Barbary doves (*Streptopelia risoria*) and white storks (*Ciconia ciconia*) all contain detectable quantities of protoporphyrin despite being immaculate and white to the human eye, while the immaculate eggshells of black-footed penguins (*Spheniscus demersus*) and common wood pigeons (*Columba palumbus*) contain both protoporphyrin and biliverdin [59]. The presence of these pigments in immaculate eggshells further brings into question the true function of these pigments, while confirming that one single hypothesis for the role of pigmentation is unlikely to explain its diversity, presence and absence in eggshells.

### 4.3. Surface kurtosis ( $S_{ku}$ )

Tyler [60] noted that in the eggshells of divers (Gaviidae), the surface resembled something akin to flat hillocks with valleys in between, with the brown pigment spots giving a rough overall appearance to the shell where pigment spots were present on the outer surface. Tyler also observed, however, that these pigment spots were not just on the outer surface of the shell, but rather were found throughout the shell itself, at depths that would be invisible from just looking at the outer surface. The presence of brown—assumably protoporphyrin—spots within the shell itself raises questions

about their function. For example, we found that species which nest close to water have higher foreground (pigment)  $S_{ku}$  compared to background. It could be hypothesized that this higher  $S_{ku}$  may contribute to preventing water from clogging up pores and generally making the surface dirty, i.e. increase hydrophobicity. However, if the pigment spots are occurring throughout the entirety of the shell—not just the outer surface—this suggests that the function goes beyond just contact with water or detritus. A fruitful further investigation (see Future directions, below) would be to measure the properties of those pigment spots contained within the shell itself, and determine if their properties match that of the outer surface pigment spots. If the surface properties of those pigment spots within the shell match that of the outer surface, it suggests they perform a similar function, but that this function is unlikely to be related to water or detritus on the outer shell surface. Such functions for internal pigment spots are more likely to be linked to thermoregulation [2,61]. For example, Maurer *et al.* [61] demonstrated that darker pigment spots acted as a form of ‘sunblock’, reducing the transmission of potentially harmful UV rays through the shell, protecting the developing embryo. Pigment spots on both the outer surface and those contained within the shell can both act to reduce UV transmission. If, however, upon further investigation, the properties of the outer surface pigment and inner pigments spots are different, this suggests that (i) while appearing visibly similar, pigment spots can have differing structural properties and (ii) the functions of pigment spots are, as has been previously suggested (e.g. [2]), not mutually exclusive. In this instance, pigment spotting contained within the shell could act as UV shielding and increasing structural integrity, while pigment spots on the outer shell surface, with higher  $S_{ku}$  than the background base colour, may act to reduce adhesion of detritus, water and, in turn, potential harmful microbes.

Prior work on eggshell nanostructures and cuticle functionality highlighted the role that risk of microbial infection and caking of the eggshell in detritus plays in determining eggshell surface properties. For example, eggs of species that typically nest in damp and humid environments exhibit higher rates of gas exchange under standard conditions than those of species which nest in drier environments [32], and have cuticular nanospheres present on the outer surface of the eggshell [62]. These cuticular nanospheres prevent the accumulation of water on the eggshell surface, helping protect the egg from bacterial and microbial infection. Interestingly, in the present study, we found that the eggs of birds who were found in regions which experienced relatively high levels of precipitation showed the opposite trend in  $S_{ku}$  to those birds which nest close to or near water. Birds inhabiting areas with high precipitation showed a higher background base-colour  $S_{ku}$  to the pigmented foreground regions. For methodological reasons, we were unable to measure relative foreground–background  $S_a$ ,  $S_{ku}$  and  $S_{sk}$  in species whose eggs were heavily maculated, as it was not possible to differentiate between background base colour and pigment spotting. Therefore, for those species we did measure that exhibit maculation, the background base-colour was the dominant surface. Thus, it is possible that a higher  $S_{ku}$  for the background has positive advantages for dealing with heavy precipitation; the same pattern was observed in  $S_{sk}$ . As  $S_{ku}$  describes the ‘peakedness’ of the surface topography, it is possible that a background eggshell surface

structure where the  $S_{ku}$  is higher assists with dealing with the higher precipitation.

#### 4.4. Future directions

While our study measured 1838 eggs from 486 species, understanding how  $S_a$ ,  $S_{sk}$  and  $S_{ku}$  vary between background and foreground eggshell surfaces across a wider selection of species would be beneficial. Moreover, studying the eggshells of species with maculated eggs that have a large-scale global distribution across multiple environmental gradients would help decode the functional reasons behind eggshell surface properties, and the relationship between foreground and background  $S_a$ ,  $S_{sk}$  and  $S_{ku}$ . Such species could include peregrine falcons (*Falco peregrinus*), ospreys (*Pandion haliaetus*), house sparrows (*Passer domesticus*) and barn swallows (*Hirundo rustica*) which have extensive distributions, spanning multiple environmental gradients. Establishing whether the relationships identified between foreground and background  $S_a$ ,  $S_{sk}$  and  $S_{ku}$  in the present study are consistent—or not—across differing environments but within species would help determine the likely function of these background/foreground differences.

Eggshell properties are known to be a labile trait [63]. For example, studies focusing on museum collections demonstrated that the eggs of reed warblers (*Acrocephalus scirpaceus*) were brighter in years with higher rainfall, and were bluer and greener in colder years [64]. Such studies suggest that changes in colour have a structural function, and may aid in thermoregulation and dissipation of water. In the present study we used eggs with limited temporal data associated with them, particularly those eggshells from the destructive collection at the Natural History Museum in Tring (e.g. [61]). Moving forward, eggshells in museum collections with date information could be used to determine how the intra-specific relationships between background and foreground  $S_a$ ,  $S_{sk}$  and  $S_{ku}$  change over time in response to different wet seasons and broader scale climate changes. Similarly, determining the consistency of the relationships between background and foreground  $S_a$ ,  $S_{sk}$  and  $S_{ku}$  within clutches would be valuable. For example, the last egg to be laid within a clutch is typically distinct from the others due to different patterning of the maculation [2]. Thus, how these differences in the degree of maculation manifest in structural properties would be important, and suggests the last egg may be different in its properties.

Comparatively little is currently known about how pigment deposition varies between species [8], and the potential implications this may have on textural properties. Whether protoporphyrin or biliverdin is deposited deeper within the shell could likely influence the difference in  $S_a$ , for example,

between the foreground and background. Moreover, while many studies work on the assumption that eggshell colour is derived from just these two pigments, recent studies on the eggshells of tinamous (Tinamidae) discovered the presence of two additional pigments: uroerythrin and bilirubin [65]. These two pigments likely offer different potential structural functions beyond the currently proposed functions of protoporphyrin and biliverdin. These pigments may contribute to the glossy nature of the eggs of tinamous, for example, in conjunction with nanostructured surface calcite and calcium phosphate crystals [15]. Hamchand *et al.* [65] noted that uroerythrin and bilirubin were photodegradable, suggesting these pigments have additional functions beyond what has been proposed for protoporphyrin. Thus, moving forward, a greater number of species require their pigment type to be determined, and their respective quantities extracted, rather than assuming only protoporphyrin and biliverdin are present.

Lastly, studies showing that the location of pigment spots can, for some species, mark areas of thinner eggshell due to lower calcium availability [1,8] warrants further investigation with respect to eggshell surface properties. While this phenomenon has only been documented in a few species, experimental manipulation of calcium availability would provide the opportunity to ascertain if pigment spots present on regions of thinner eggshell due to low calcium availability differ in their  $S_a$ ,  $S_{sk}$  and  $S_{ku}$  properties in comparison to pigment spots present on eggshells produced in areas with plentiful calcium availability.

**Data accessibility.** The data and code used for statistical analysis are available at: <https://figshare.com/s/cb5e8588944af1ea529>. Statistical outputs are provided in the electronic supplementary material.

The data are provided in electronic supplementary material [66].

**Authors' contributions.** M.R.G.A.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing—original draft, writing—review and editing; J.B.: conceptualization, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing—review and editing; S.J.P.: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

**Conflict of interest declaration.** The authors declare no conflict of interest.

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