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Author for correspondence:
Daniel Hanley
e-mail: danielhanley00@gmail.com

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Not so colourful after all: eggshell pigments constrain avian eggshell colour space

Daniel Hanley[1], Tomáš Grim[1], Phillip Cassey[2] and Mark E. Hauber[3]

[1]Department of Zoology and Laboratory of Ornithology, Palacký University, 17. listopadu 50, Olomouc 77146, Czech Republic
[2]School of Biological Sciences, University of Adelaide, Adelaide, South Australia 5005, Australia
[3]Department of Psychology, Hunter College and the Graduate Center, The City University of New York, 695 Park Avenue, New York, NY 10065, USA

Birds' eggshells are renowned for their striking colours and varied patterns. Although often considered exceptionally diverse, we report that avian eggshell coloration, sampled here across the full phylogenetic diversity of birds, occupies only 0.08–0.10% of the avian perceivable colour space. The concentrations of the two known tetrapyrrole eggshell pigments (protoporphyrin and biliverdin) are generally poor predictors of colour, both intra- and interspecifically. Here, we show that the constrained diversity of eggshell coloration can be accurately predicted by colour mixing models based on the relative contribution of both pigments and we demonstrate that the models' predictions can be improved by accounting for the reflectance of the eggshell's calcium carbonate matrix. The establishment of these proximate links between pigmentation and colour will enable future tests of hypotheses on the functions of perceived avian eggshell colours that depend on eggshell chemistry. More generally, colour mixing models are not limited to avian eggshell colours but apply to any natural colour. Our approach illustrates how modelling can aid the understanding of constraints on phenotypic diversity.

1. Introduction

Birds' eggshells display a variety of colours and striking patterns that have captured the attention of philosophers, artists and scientists since the time of Aristotle [1]. The diversity of colour is generally attributed to biliverdin IXα, appearing blue–green, and protoporphyrin IX, appearing rusty-brown [2]. There is strong evidence that eggshell colours and their physical–chemical bases are adaptive in many contexts [3].

Contrary to dietary sources of avian coloration (e.g. carotenoids, as found in birds' feathers), biliverdin and protoporphyrin are synthesized pigments [4,5]. One limitation to understanding the function of eggshell coloration is the unresolved relationship between pigment concentrations and their perceived colours. While some studies have found correlations between pigment concentrations and eggshell coloration within species [6,7], others have not found these patterns within [8] or among species for either ground coloration [2] or maculation patterns [9]. However, such a quantitative link between variation in eggshell pigmentation and avian-perceived variation in eggshell colour is fundamental for testing evolutionary and functional hypotheses.

Here, we integrate empirical and model-based approaches to examine avian-perceived eggshell colours. We generate predicted colours using two subtractive colour mixing models that each combined different components of eggshell colour [10]. First, we mixed the colours of a purely biliverdin-pigmented eggshell and a purely protoporphyrin-pigmented eggshell (hereafter 'simple model'). Second, we then additionally mixed the colour of

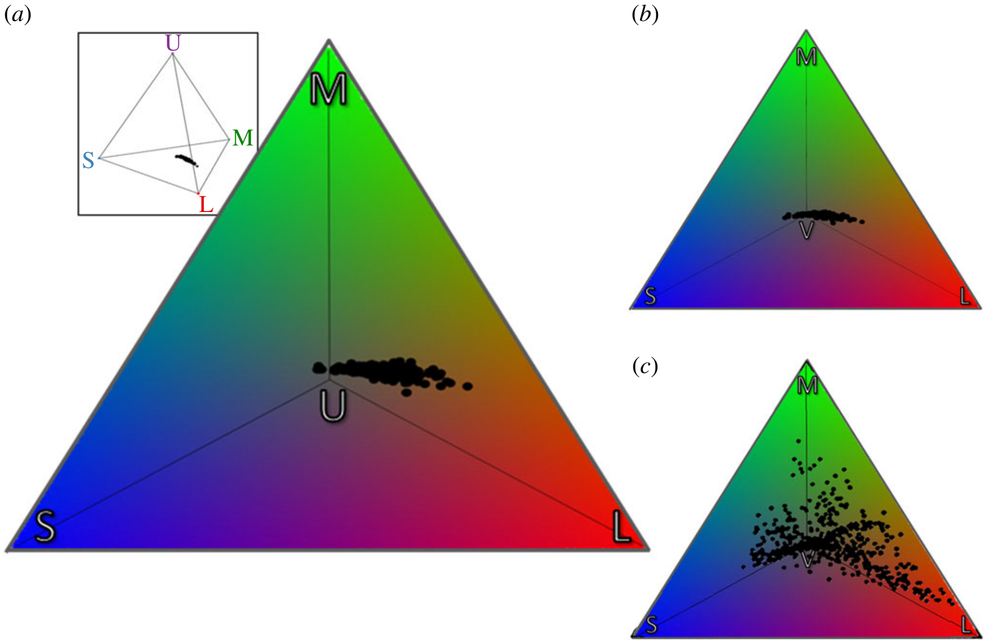

Figure 1. The distribution of (*a*) birds' eggshell colours (this study) within the ultraviolet-sensitive (UVS) avian tetrahedral colour space (inset) when viewed under daylight conditions. We compared the perceptual spaces occupied by (*b*) avian eggshell colours with (*c*) avian feather colours (sourced and adapted from [12]) in the violet-sensitive (VS) avian colour space as they were originally presented using 'a standard constant illumination across all visible wavelengths' *sensu* [12]. The plots illustrate the stimulation of the short (S), medium (M), long (L), and either (*a*) ultraviolet (U) or (*b*,*c*) violet (V) wavelength-sensitive photoreceptors. All plots are shown from above the U or V vertex of the tetrahedral colour space. (Online version in colour.)

an unpigmented eggshell, representing a pure calcium carbonate eggshell matrix (hereafter 'general model'). Using eggs representing the full phylogenetic diversity of birds (electronic supplementary material, figure S1), we tested whether these 'model-predicted' eggshell colours encompassed the entire avian eggshell colour gamut (i.e. the complete range of avian-perceivable eggshell colours).

2. Material and methods

(a) Colour analyses

We used the average reflectance spectra of avian eggshells stored in natural history museums (figure 1*a*) from 636 species (electronic supplementary material, figure S1) originally collected by Hanley *et al.* [11] (for further details, see electronic supplementary material). We calculated avian-perceived variation in colour using receptor-noise-limited models [13] accounting for the visual sensitivity of the average ultraviolet-sensitive (UVS) or violet-sensitive (VS) avian receivers [14], the double cone sensitivity of the blue tit, *Cyanistes caeruleus*, and domestic chicken, *Gallus gallus*, respectively, and irradiance spectra (scaled by 10 000) representing bright illumination under direct daylight and filtered forest light viewing conditions. These calculations generated values that represented the relative stimulation of birds' four single cones and double cones (electronic supplementary material, table S1). We converted these values into spatial coordinates within the UVS and VS avian tetrahedral colour spaces (hereafter 'natural eggshell colours'). The avian tetrahedral colour space removes achromatic information; however, chromatic and achromatic variation is thought to be perceived via separate mechanisms in birds [12]. Colour analyses were conducted using the 'pavo' software package [15].

(b) Comparing pigment mixing model outputs with the range of natural eggshell colours

Based on the spectra for two eggshells, each containing only a single pigment, 100 intermediate reflectance spectra were generated. These intermediate spectra were derived using a Yule–Nielsen subtractive colour mixing model [10] as follows:

$$\text{predicted } R_\lambda = \prod_{i=1}^{N_c} R_{i,\lambda}^{c_i} \tag{2.1}$$

where N_c represents the number of colorants, R represents the reflectance at each wavelength (λ) and c represents the relative concentration such that the sum of all relative concentrations equals 1. Here, the American robin (*Turdus migratorius*: electronic supplementary material, table S2) was used as a purely biliverdin-based eggshell [2] and the peregrine falcon (*Falco peregrinus*: electronic supplementary material, table S2) as a purely protoporphyrin-based eggshell [2].

Next, we also incorporated the spectral characteristics of the calcium carbonate eggshell matrix into the subtractive model (the 'general model': figure 2*c*), by including the reflectance of an immaculate white Northern fulmar (*Fulmarus glacialis*: electronic supplementary material, table S2) eggshell, representing an unpigmented eggshell [2]. We again generated 100 intermediate reflectance spectra (figure 2*c*). The predictive ability of each model was examined with three approaches: we compared the overlap between the actual and model-generated colour spaces, we determined how close the model-generated colours were to the line natural eggshell colours formed through three-dimensional visual space (hereafter 'absolute residual'), and we calculated how dispersed the *x*-coordinates of the model-generated colours were relative to the full range of the avian eggshell colour gamut (for further details, see electronic supplementary material). Using different species to represent purely pigmented or unpigmented eggshells did not change our conclusions (electronic supplementary material).

3. Results

Avian eggshell colours occupied very little (less than 1%) of the UVS avian-perceivable colour space: 0.09% in daylight (figure 1*a*), and 0.08% in forest light conditions. Similarly,

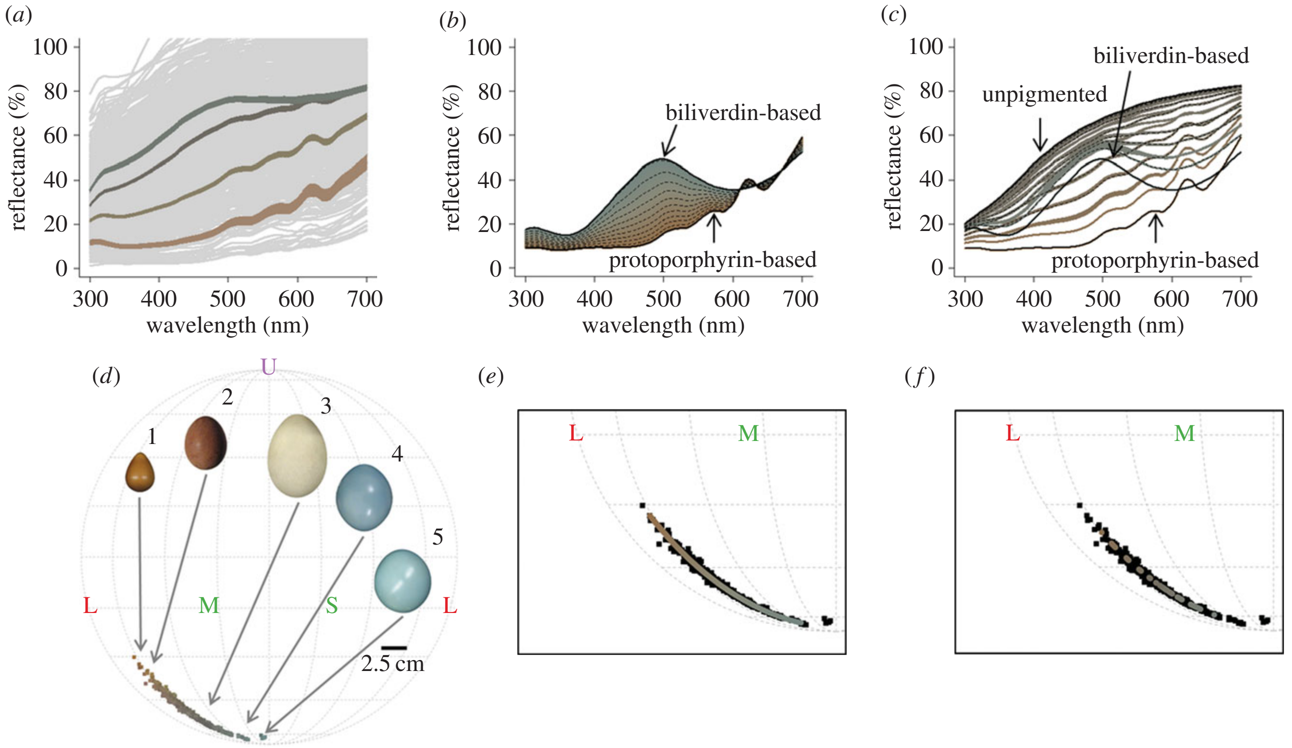

Figure 2. The reflectance spectra of (*a*) all avian eggshells from [11] (grey), summarized by four *k*-means clusters (means ± s.e.; plotted in their actual colours), (*b*) the simple model's output and (*c*) the general model's output with reflectance spectra of pure/no pigments (solid black lines), every 10th spectrum (dashed lines), and all intermediate spectra (full colour shading). We illustrate a Mollweide projection of the hue distribution of (*d*) natural eggshell colour in UVS avian colour space, plotted in the actual colours that maintained their relative brightness, with five representative eggs: (1) *Hydrophasianus chirurgus* (FMNH 15312), (2) *Falco peregrinus* (UMMZ 231817), (3) *Fulmarus glacialis* (FMNH 4913), (4) *Tinamus major* (UMMZ 191600) and (5) *Tinamus osgoodi* (FMNH 2856). The letters represent the ultraviolet (U), short (S), medium (M) and long (L) wavelength-sensitive photoreceptors. We depict the (*e*) simple and (*f*) general model outputs' hue distributions above natural eggshell colours (black). (Online version in colour.)

eggshell colours occupied only 0.10% (figure 1*b*) of the VS avian-perceivable colour space in daylight conditions, and 0.08% of the colour space in forest light conditions.

Both the simple and general models generated colours that fell completely (100%) within the natural eggshell colour gamut. However, the simple model output did not match natural eggshell colours as accurately as randomly sampled natural eggshell colours matched themselves (hereafter 'null model'; $t = 21.26$, d.f. $= 150.53$, $p < 0.0001$; electronic supplementary material, figure S2). By contrast, the general model output matched natural egg colours better than randomly selected natural egg colours matched themselves ($t = -16.36$, d.f. $= 197.07$, $p < 0.0001$; electronic supplementary material, figure S2), which was a substantial improvement over the output of the simple model ($t = -30.11$, d.f. $= 136.50$, $p < 0.0001$). All colours from the simple, general and null models had significantly smaller (all $p < 0.0001$) absolute residuals than points randomly drawn from the UVS avian colour space (electronic supplementary material, figure S2).

The dispersion of the x-coordinates of the colours generated by the simple model represented 76% of the dispersion of natural eggshell colours (figure 2*e*). The general model produced colours that were 54% as dispersed as natural eggshell colours (figure 2*f*).

4. Discussion

Given the continued and widespread scientific and aesthetic interest in colourful avian eggshells, and the traditional awe over their diversity, the avian eggshell colour gamut is surprisingly small. In fact, to a bird's eyes, their eggs are 200- to 400-times less diverse in colour than their feathers (this study versus [12]; figure 1*b*,*c*). Additionally, we document that variation in avian eggshell colour is directly associated with the relative contribution of biliverdin and protoporphyrin, particularly when accounting for their integration within a calcium carbonate matrix of the eggshell.

Both sets of model-generated colours were within the avian eggshell colour gamut and varied along the same axis of variation as real eggshells. We found that the simple model-generated colours more thoroughly covered the entire range of natural eggshell colours (i.e. dispersion: figure 2*e*,*f*), but the general model-generated colours more accurately matched the spectral reflectance of natural eggshell colours (figure 2*c*; electronic supplementary material, figure S2). Nonetheless, these models cannot yet predict the limits of eggshell colour diversity because the colours of some natural eggshells, with unknown pigment concentrations, fall outside the model-predicted ranges (figure 2*e*,*f*). Currently, our models also assume an even mixing of the pigments throughout the eggshell, but in some species pigment concentrations vary across the eggshell layers [16]; therefore, further analyses are required for such species. Future research explicitly interested in eggshell appearance should consider ground coloration (as we did), luminance and eggshell patterning.

Just as with birds' feathers [12], avian eggshell colours should be limited within the proximate limits set by colour production mechanisms and the ultimate limits set by selective pressures. Variation in the colours of birds' feathers is mostly attributable to structural colour, with pigments contributing little to the colour diversity (approx. 7% of the total 26% of the VS colour space occupied by feather colours) [12]; in feathers, individual pigment classes occupy very little of avian perceptual colour space indeed, from 0.1% for porphyrins to 3.5% for carotenoids [12]. Just as with tetrapyrrole feather pigments (turacin and turacoverdin) [12], our models predict that tetrapyrrole eggshell pigments (protoporphyrin and biliverdin) occupy very little of avian colour space (approx. 0.10%).

Our evidence supports chemical analyses [17] that found just two pigments responsible for birds' eggshell colours and implies that structural or other factors are only minor contributors to avian eggshell coloration [18,19]. The constraint in perceivable chromatic variation may suggest the relative importance of the achromatic component of eggshell colour or suggest alternative non-visual functions for eggshell pigments [3]. These colour mixing models can be applied to any natural colour, and, more generally, they demonstrate a novel approach to understanding trait diversity. This study enables future exploration of the expression and constraint of avian eggshell coloration by establishing a direct link between pigmentation and avian-perceived eggshell colours.

Ethics. No live animals were studied.

Data Accessibility. Reflectances: http://dx.doi.org/10.5061/dryad.2q3r2.

Authors' Contribution. D.H. and M.E.H. conceived the study; D.H. and P.C. collected the data; D.H., M.E.H. and T.G. planned the analyses; D.H. generated the models and ran the analyses; and D.H., P.C., T.G. and M.E.H. wrote the manuscript. All authors approved publication.

Competing Interests. We declare we have no competing interests.

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References

1. Aristotle. 350 BC *The history of animals*. Boston, MA: Internet Classics Archive, MIT.
2. Cassey P, Thomas GH, Portugal SJ, Maurer G, Hauber ME, Grim T, Lovell PG, Mikšík I. 2012 Why are birds' eggs colourful? Eggshell pigments co-vary with life-history and nesting ecology among British breeding non-passerine birds. *Biol. J. Linn. Soc.* **106**, 657–672. (doi:10.1111/j.1095-8312.2012.01877.x)
3. Cassey P, Maurer G, Lovell PG, Hanley D. 2011 Conspicuous eggs and colourful hypotheses: testing the role of multiple influences on avian eggshell appearance. *Avian Biol. Res.* **4**, 185–195. (doi:10.3184/175815511X13207699868421)
4. Baird T, Solomon SE, Tedstone DR. 1975 Localisation and characterisation of egg shell porphyrins in several avian species. *Br. Poult. Sci.* **16**, 201–208. (doi:10.1080/00071667508416177)
5. Zhao R, Xu GY, Liu ZZ, Li JY, Yang N. 2006 A study on eggshell pigmentation: biliverdin in blue-shelled chickens. *Poult. Sci.* **85**, 546–549. (doi:10.1093/ps/85.3.546)
6. Lopez-Rull I, Miksik I, Gil D. 2008 Egg pigmentation reflects female and egg quality in the spotless starling *Sturnus unicolor*. *Behav. Ecol. Sociobiol.* **62**, 1877–1884. (doi:10.1007/s00265-008-0617-1)
7. Moreno J, Lobato E, Morales J, Merino S, Tomas G, Martinez-de la Puente J, Sanz JJ, Mateo R, Soler JJ. 2006 Experimental evidence that egg color indicates female condition at laying in a songbird. *Behav. Ecol.* **17**, 651–655. (doi:10.1093/beheco/ark014)
8. Cassey P *et al*. 2012 Avian eggshell pigments are not consistently correlated with colour measurements or egg constituents in two *Turdus* thrushes. *J. Avian Biol.* **43**, 503–512. (doi:10.1111/j.1600-048X.2012.05576.x)
9. Brulez K, Cassey P, Meeson A, Mikšík I, Webber SL, Gosler AG, Reynolds SJ. 2014 Eggshell spot scoring methods cannot be used as a reliable proxy to determine pigment quantity. *J. Avian Biol.* **45**, 94–102. (doi:10.1111/j.1600-048X.2013.00236.x)
10. Simonot L, Hébert M. 2014 Between additive and subtractive color mixings: intermediate mixing models. *J. Opt. Soc. Am. A* **31**, 58–66. (doi:10.1364/JOSAA.31.000058)
11. Hanley D, Cassey P, Doucet SM. 2013 Parents, predators, parasites, and the evolution of eggshell colour in open nesting birds. *Evol. Ecol.* **27**, 593–617. (doi:10.1007/s10682-012-9619-6)
12. Stoddard M, Prum R. 2011 How colorful are birds? Evolution of the avian plumage color gamut. *Behav. Ecol.* **22**, 1042–1052. (doi:10.1093/beheco/arr088)
13. Vorobyev M, Osorio D. 1998 Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. Lond. B* **265**, 351–358. (doi:10.1098/rspb.1998.0302)
14. Endler JA, Mielke PW. 2005 Comparing entire colour patterns as birds see them. *Biol. J. Linn. Soc.* **86**, 405–431. (doi:10.1111/j.1095-8312.2005.00540.x)
15. Maia R, Eliason CM, Bitton P, Doucet SM, Shawkey MD. 2013 pavo: an R package for the analysis, visualization and organization of spectral data. *Methods Ecol. Evol.* **4**, 906–913. (doi:10.1111/2041-210X.12069)
16. Liu HC, Hsiao MC, Hu YH, Lee SR, Cheng WTK. 2010 Eggshell pigmentation study in blue-shelled and white-shelled ducks. *Asian Australas. J. Anim. Sci.* **23**, 162–168. (doi:10.5713/ajas.2010.90256)
17. Gorchein A, Lim CK, Cassey P. 2009 Extraction and analysis of colourful eggshell pigments using HPLC and HPLC/electrospray ionization tandem mass spectrometry. *Biomed. Chromatogr.* **23**, 602–606. (doi:10.1002/bmc.1158)
18. Igic B *et al*. 2015 A nanostructural basis for gloss of avian eggshells. *J. R. Soc. Interface* **12**, 20141210. (doi:10.1098/rsif.2014.1210)
19. Fecheyr-Lippens DC, Igic B, D'Alba L, Hanley D, Verdes A, Holford M, Waterhouse GIN, Grim T, Hauber ME, Shawkey MD. 2015 The cuticle modulates ultraviolet reflectance of avian eggshells. *Biology Open* (doi:10.1242/bio.012211)

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Supplementary Methods

(a) Eggshell reflectance measurements

The details of the methods for collecting the data used for this study were previously described in detail [1], and are therefore only briefly outlined here. We collected reflectance spectra from 5,604 eggshells from 636 species (mean ± SE: 3.06 ± 0.07 clutches per species, 8.81 ± 0.27 eggs per species) representing all avian orders (figure S1) except for sand grouse (Pterocliformes). The eggshell specimens used in this study were stored at the American Museum of Natural History (New York, USA), the Field Museum (Chicago, USA), the University of Michigan Museum of Zoology (Ann Arbor, USA), and the Natural History Museum at Tring (Tring, UK). We collected six spectra for each eggshell by taking two measurements from three distinct regions of the eggshell: the blunt end, the equator, and the pointed end [2], avoiding eggshell spots. Each spectrum (N = 33,624) was visually inspected and we excluded aberrant spectra (N = 29) prior to averaging by egg, then by clutch and then by species.

The measurement protocol varied slightly between museums. Specifically, we used a coincident normal measurement angle to measure the eggshells from the Natural History Museum at Tring (31% of eggshells sampled) and used an Ocean Optics USB2000 Miniature Fibre Optic Spectrometer illuminated by a DT mini lamp [2]. To avoid specular glare from glossy eggshells, the remaining eggshells were measured with a 45-degree coincident oblique measurement geometry using an Ocean Optics USB 4000 and a PX-2 pulsed xenon light source (Ocean Optics, Dunedin, FL). In both measurement protocols, we each used a Spectralon™ white standard (WS-1; Ocean Optics, Dunedin, FL).

General linear mixed models were reported [1] to examine how colour was related to species and museum for data from 25 species that were measured using both measurement procedures. If the difference in measurement angle influenced colour measurements, then the measurements taken with a coincident normal measurement angle would have differed from all those taken at a 45-degree coincident oblique measurement geometry. These analyses revealed that the variation attributable to species was far greater than that attributed to museum, and that the measurements taken with different measurement geometries were always statistically similar [1]. Therefore, we pooled these data and used their species average values. We used these spectra to perform further colour analyses and to generate avian visual models with the ‘pavo’ software package [3].

We calculated avian perceived variation in colour using receptor-noise limited models [4]. These models accounted for the visual sensitivity of the average ultraviolet sensitive (UVS) or violet sensitive (VS) avian receivers [5]. We also modelled the double cone sensitivity of the blue tit *Cyanistes caeruleus* and domestic chicken *Gallus gallus* for UVS and VS avian receivers, respectively. We used two types of irradiance spectra that represented direct daylight or filtered forest light (both scaled by 10,000) under bright viewing conditions. These calculations generated values relative quantum catches for birds’ four single cones and double cones (table S1). We then transformed these values into coordinates within the UVS and VS avian tetrahedral colour spaces [e.g., 6].

(b) Calculating how much calcium carbonate to include in the general model

The simple model (see main text) combines only two reflectance spectra representing variable contributions of a purely biliverdin-pigmented eggshell and a purely protoporphyrin-pigmented eggshell. However, more colours can be mixed using eq. 1.

$$\text{predicted } R_\lambda = \prod_{i=1}^{N_c} R_{i,\lambda}^{c_i} \qquad \text{eq. 1}$$

The general model enhances the simple model by adding the reflectance spectrum of a white avian eggshell, representing the colour of calcium carbonate. To use this function, we must know the reflectance at each wavelength ($R_{i,\lambda}$) for each colourant (N_c) and that colourant’s concentration (c_i). However, we do not know the relative amount of calcium carbonate that should be mixed to accurately colour match avian eggshells; therefore, this must be estimated for each model. We used three steps to estimate the relative amount of calcium carbonate needed to mix with pigment contributions.

First, the relative and absolute concentration of whichever of the two pigments was more concentrated in the eggshell was scaled between 0 and 1 using a dose-dependent *function,*

$$f(x) = 1 - \left[1 + \frac{x}{\beta}\right]^{-\alpha} \qquad \text{eq. 2}$$

and these scaled factors were then subtracted from 1 to represent the contribution not attributable to these pigments. Second, to account for the combined influence of these scaled relative and absolute pigment concentrations (obtained from eq. 2), we calculated their weighted mean using a scaling factor, S (where S represents the relative concentration and $1-S$ represents the absolute concentration). Finally, we used a Poisson distribution function to predict the relative concentration of calcium carbonate based on these mean values, such that the remainder of the colour was attributed to both eggshell pigments. We then again used eq. 1 to predict a reflectance spectrum, this time mixing the variable contribution of three input colours rather than two.

(c) Optimising our general model

To optimise the parameters of our general model, we surveyed species with published eggshell pigment concentrations and numerically matched the models' predicted reflectance spectra with their actual reflectance spectra [7] (table S2). Specifically, we allowed each parameter to vary within a set of values and examined the difference between actual and predicted reflectance for all combinations of these sets of parameter values (N = 1,293,600). Due to their different scales and an initial exploratory analysis, we used different α values for the relative and absolute concentrations. For relative concentration we optimised α within the set {0, 1, 2, …, 15}, while for absolute concentration α was optimised within the set {0, 0.05, 0.10, …, 1}. We also searched for the optimal β in the set {0, 1, 2, …, 6}, S in the set {0, 0.1, 0.2, …, 1}, and the λ value for the Poisson distribution in the set {1, 2, 3, …, 50} (please note that, as stated above, λ in eq. 1 refers to the wavelength for reflectance spectra, while this lambda refers to the λ for the Poisson distribution). We used the combination of parameter values that resulted in the smallest difference between the predicted and the actual reflectance spectra. These values were 4 for α attributed to the relative concentration, 0.8 for the α attributed to absolute concentration, 1 for β, 0.9 for the scaling factor S (i.e., we weighted the two scaled factors such that the relative concentration accounted for 90% of the weighted mean), and 7 for the λ value for the Poisson distribution. This optimization was conducted in R version 3.0.3 (R Development Team 2014), using the high performance cluster provided by MetaCentrum/CERIT-SC, which is a network of computers made available by the Czech Education and Scientific Network and participating universities within the Czech Republic.

(d) Comparing predicted and observed colours

First, we calculate the exact overlap between actual and model-predicted avian eggshell colours [3], rather than a Monte Carlo approximation [*sensu* 8] to determine if our model-predicted eggshell colours fall within the avian eggshell colour gamut. Then, space using a resampling procedure, we compared the fit of our predicted data to the line that natural eggshells generate through the UVS avian visual space. We began by randomly sampling 100 natural eggshell colours. Then, we constructed a model to predict the x coordinate in UVS avian visual space, because this coordinate accounted for 92.6% of the variance in natural eggshell colours (variance of x divided by the sum of variance in x, y, and z). This model predicted the x coordinates by the y and z coordinates of natural eggshell colours using a quadratic model,

$$f\,(\mathrm{y},\mathrm{z}) = a\mathrm{y}^2+b\mathrm{z}^2+c\mathrm{yz}+d\mathrm{y}+e\mathrm{z}+f \qquad \text{eq. 3}$$

where a, b, c, d, e, and f are coefficients in the quadratic model to account for the non-linear relationship between x and y, and x and z, respectively.

We then used this model to predict the x coordinates for a separate set of 100 randomly selected natural eggshell colours and to calculate the mean absolute value of the difference between the predicted and actual x coordinates (hereafter ‘absolute residual’) from our second resample. To generate distributions of absolute residuals we repeated this 1,000 times for natural eggshell colours (null), colours generated by the simple and general models, as well as randomly selected colours from anywhere within the UVS avian colour space (random). To avoid inflating degrees of freedom, we compared 100 randomly chosen absolute residuals from these distributions (each N = 1,000) using two-sample t-test tests (figure S2*a* & S2*b*). Because we made no inter-specific comparisons, and our model-generated colours were not associated with any particular species, we did not control for phylogeny in these analyses (see also [8]). In addition, to determine how closely both sets of model-generated colours represented the full range of the avian eggshell colour gamut, we reported the dispersion of the x coordinate of natural eggshell colours and the colours predicted by our simple and general models (i.e., the ratio of the generated range to the natural range). Again, we reran these analyses using a different set of species (see above) and the results were statistically similar and did not influence the conclusions (figures S4 & S5).

(e) Why we did not control for phylogeny when comparing predicted and observed colours

We used a resampling procedure to compare the perceptual match between 100 actual eggshell colours and our model-predicted colours, and repeated this 1,000 times (for full details see main text). While these natural eggshell colours were from 100 different species, we were interested in the distribution of avian perceived eggshell colours within the birds' visible colour space (irrespective of species) [8]. In addition, because we were assessing the accuracy of model-generated colours, which are not associated with any species, and because we did not make interspecific comparisons, we did not control for phylogeny.

(f) Repeated analyses when selecting other species with sole-pigment eggshell colours

We repeated the output of our colour mixing models using two different bird species' eggshells from those reported in the main text of this study. For these analyses, we selected the great tinamou (*Tinamus major*) to represent a purely biliverdin-pigmented eggshell (table S2), the domestic chicken (*Gallus gallus domesticus*) of a brown-egg-laying breed to represent a purely protoporphyrin-pigmented eggshell (table S2), and the great crested grebe (*Podiceps cristatus*) to represent a relatively unpigmented, white eggshell (table S2). The results of these repeated analyses were statistically similar to the results presented in the main text. Briefly, we again optimised the general model parameters to accommodate these new pure-pigment endpoints and found that the optimal α for relative concentration was 9, α for absolute concentration was 0.2, β was 3, 0.9 for the scaling factor S, and the λ for the Poisson distribution was 13. The colours generated by the simple model (figure S4*a* and S4*b*) overlapped natural eggshell colours, but not as well as randomly sampled natural eggshell colours matched other randomly sampled natural eggshell colours ($t = 23.55$, $df = 111.89$, $P < 0.0001$; figure S5). However, colours that were generated by the general model (figure S4*c* and S4*d*) overlapped randomly selected natural egg colours better than randomly selected natural egg colours overlapped themselves ($t = -12.29$, $df = 154.80$, $P < 0.0001$; figure S5), which was an improvement over the output of the simple model ($t = -22.33$, $df = 129.97$, $P < 0.0001$). The x coordinates in the UVS avian visual space of colours generated by the simple model were 66% (figure S4*b*) as dispersed as those of natural eggshell colours. In contrast, the colours generated by the general model were only 43% as dispersed as natural eggshell colours (figure S4*d*). The absolute residuals of colours generated by the simple, general, and null models were significantly smaller (all $P < 0.0001$) than points randomly drawn from the UVS avian colour space (figure S5).

References

1. Hanley D, Cassey P & Doucet SM 2013 Parents, predators, parasites, and the evolution of eggshell colour in open nesting birds. *Evol. Ecol.* **27**, 593–617.

2. Cassey P, Portugal SJ, Maurer G, Ewen JG, Boulton RL, Hauber ME & Blackburn TM 2010 Variability in avian eggshell colour: a comparative study of museum eggshells. *PLoS ONE* **5**, e12054.

3. Maia R, Eliason CM, Bitton P, Doucet SM & Shawkey MD 2013 pavo: an R package for the analysis, visualization and organization of spectral data. *Methods Ecol. Evol.* **4**, 906–913.

4. Vorobyev M & Osorio D 1998 Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. London B* **265**, 351–358.

5. Endler JA & Mielke PW 2005 Comparing entire colour patterns as birds see them. *Biol. J. Linn. Soc.* **86**, 405–431.

6. Stoddard MC & Prum RO 2008 Evolution of avian plumage color in a tetrahedral color space: a phylogenetic analysis of new world buntings. *Am. Nat.* **171**, 755–776.

7. Cassey P, Thomas GH, Portugal SJ, Maurer G, Hauber ME, Grim T, Lovell PG & Mikšík I 2012 Why are birds' eggs colourful? Eggshell pigments co-vary with life-history and nesting ecology among British breeding non-passerine birds. *Biol. J. Linn. Soc.* **106**, 657–672.

8. Stoddard M & Prum R 2011 How colorful are birds? Evolution of the avian plumage color gamut. *Behav. Ecol.* **22**, 1042–1052.

9. Clements JF 2007 *The Clements checklist of birds of the world*. 6th edn. Ithaca, NY: Comstock publishing associates.

10. Mitchell KJ, Llamas B, Soubrier J, Rawlence NJ, Worthy TH, Wood J, Lee MSY & Cooper A 2014 Ancient DNA reveals elephant birds and kiwi are sister taxa and clarifies ratite bird evolution. *Science* **344**, 898–900.

11. Zuccon D & Ericson PGP 2012 Molecular Phylogenetics and Evolution Molecular and morphological evidences place the extinct New Zealand endemic *Turnagra capensis* in the Oriolidae. *Mol. Phylogenet. Evol.* **62**, 414–426.

Table S1

The median (interquartile range, represented as first – third quartile) relative stimulation for avian single and double cone types, representing perceived chromatic and achromatic variation, respectively, for natural eggshell colour measurements and predicted eggshell colours from the simple and general colour mixing models.

Table S2

The average concentrations ($\mathrm{nmol \cdot g^{-1}}$) of biliverdin IXα and protoporphyrin IX pigments extracted from birds' eggshells for select species from two published sources. We used 15 of these species (*), from a single published source that used a consistent sampling protocol [7], to optimize our general model.

Figure S1

A representative phylogeny depicting the orders represented in our eggshell colour database, based on Clement's Checklist [9]. This phylogeny was created using taxonomic and molecular data provided by http://birdtree.org, and two extinct taxa (*Aepyornis maximus* and *Turnagra capensis*) were added based on recent molecular evidence [10,11].

Figure S2

We display the frequencies of 1000 resampled absolute residuals (see main text) of the (a) colours predicted by the general model (general), from 100 randomly sampled natural eggshell colours (null), from the colours predicted by the simple model (simple), and (b) from 100 randomly selected coordinates within the full UVS avian colour space (random).

Figure S3

The absolute difference between actual (solid) reflectance spectra of avian eggshells, and those predicted by the simple (dotted) and general models (dash) for selected species with known pigment concentration.

Figure S4

The reflectance spectra of (a) the simple model output (mixing biliverdin- and protoporphyrin-based colours only; natural eggshell colour is plotted in solid black), and the (b) UVS avian hue distributions for these model generated colours, superimposed above the natural eggshell colours (black). We also show the (c) output of the general model (mixing biliverdin-, protoporphyrin-, and calcium carbonate-based colours; natural eggshell colour is plotted in solid black), and the associated UVS avian hue distributions for these colours. For each set of reflectance spectra we depict spectra based on pure pigments (solid black lines), every 10th spectra (dashed lines), and all intermediate spectra (full colour shading). Hue distributions are plotted as Mollweide projections and the letters inside the coordinate system represent the photoreceptor types (U = UVS, S = SWS, M = MWS, L = LWS). Compare with Figure 2 from the main manuscript.

Figure S5

The frequencies of absolute residuals from the general (general), null (null), and simple (simple) models as well as from a model with randomly selected coordinates within the UVS avian colour space (random). Note that the scale of the x and y axes for random coordinates are different. For further details see the Methods and figure 2 from the main text.

Table S1

Perception	Photoreceptor type	Natural	Simple	General
Chromatic	Ultraviolet sensitive	0.05 (0.04–0.05)	0.03 (0.03–0.03)	0.04 (0.04–0.04)
Chromatic	Short-wave sensitive	0.25 (0.22–0.26)	0.21 (0.17–0.25)	0.24 (0.23–0.24)
Chromatic	Medium-wave sensitive	0.34 (0.33–0.34)	0.34 (0.33–0.34)	0.34 (0.34–0.34)
Chromatic	Long-wave sensitive	0.37 (0.35–0.40)	0.42 (0.37–0.47)	0.38 (0.38–0.39)
Achromatic	Done cone	0.46 (0.34–0.58)	0.81 (0.72–0.91)	0.79 (0.79–0.82)

Table S2

Species	**Biliverdin IXα (nmol·g^{-1})**	**Protoporphyrin IX (nmol·g^{-1})**	**Reference**
Tinamus major	97.12	0.00	2
Gallus gallus	0.00	9.27	2
*Anas platyrhynchos**	0.14	3.98	1
*Somateria mollissima**	0.26	8.86	1
*Podiceps cristatus**	0.74	1.18	1
*Apus apus**	3.77	21.28	1
*Otis tarda**	4.27	20.42	1
*Gallinula chloropus**	2.36	17.02	1
*Vanellus vanellus**	69.28	478.06	1
*Sterna sandvicensis**	23.48	213.77	1
*Gavia arctica**	0.26	78.87	1
*Fulmarus glacialis**	0.04	0.36	1
*Pandion haliaetus**	0.10	12.04	1
*Falco peregrinus**	0.00	81.84	1
*Upupa epops**	1.69	45.80	1
*Alcedo atthis**	8.62	34.69	1
*Merops apiaster**	18.97	70.94	1
Turdus migratorius	4.46	0.00	2

1. Cassey P, et al. (2012) Why are birds' eggs colourful? Eggshell pigments co-vary with life-history and nesting ecology among British breeding non-passerine birds. *Biol J Linn Soc* 106(3):657–672.

2. Verdes A (2014) Nature's Palette. Master's Thesis in Biology, Department of Biological Sciences, Hunter College (CUNY).

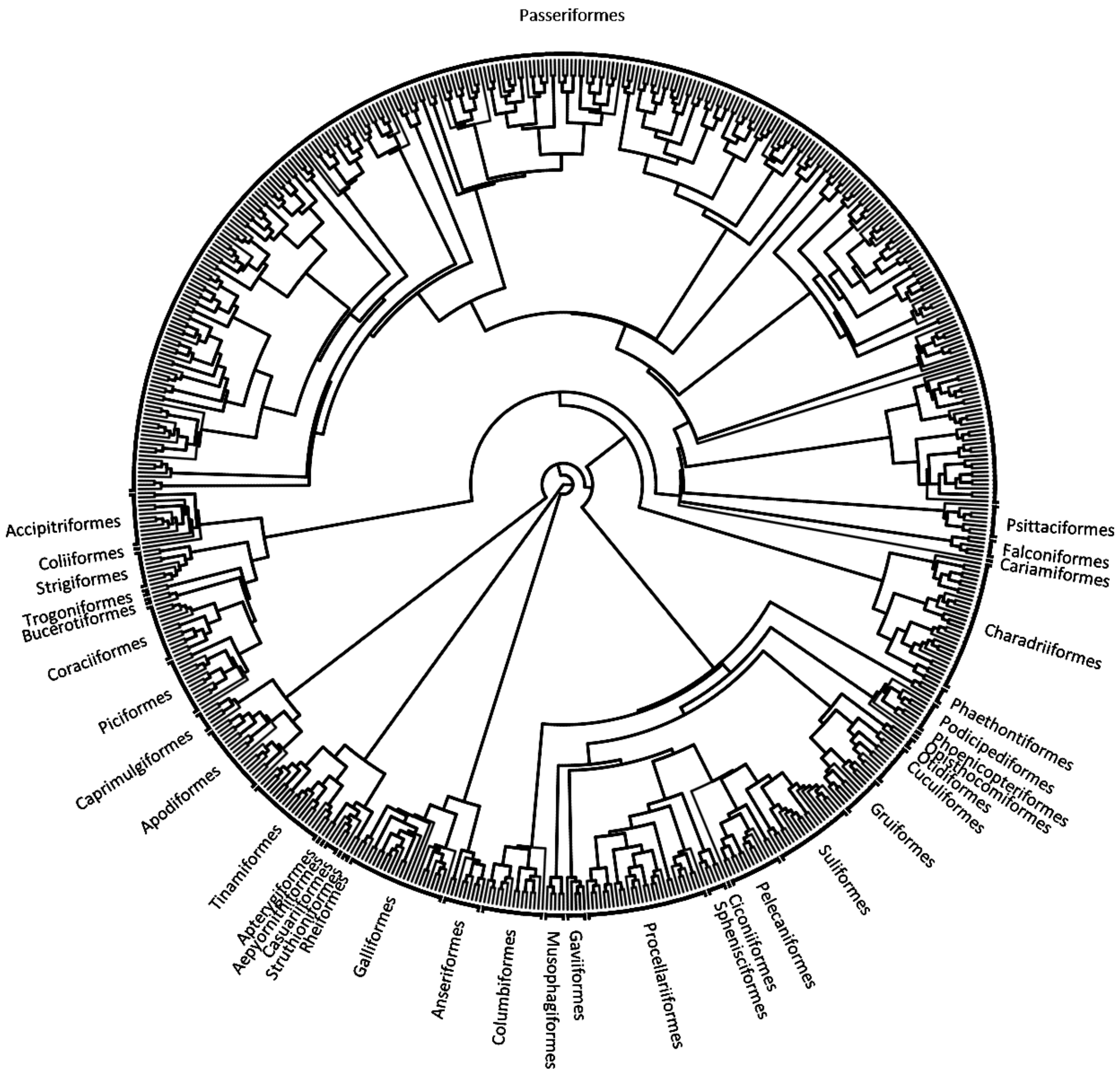
Passeriformes
Psittaciformes
Falconiformes
Cariamiformes
Charadriiformes
Phaethontiformes
Podicipediformes
Phoenicopteriformes
Opisthocomiformes
Otidiformes
Cuculiformes
Gruiformes
Suliformes
Pelecaniformes
Ciconiiformes
Sphenisciformes
Procellariiformes
Gaviiformes
Musophagiformes
Columbiformes
Anseriformes
Galliformes
Rheiformes
Struthioniformes
Casuariiformes
Aepyornithiformes
Apterygiformes
Tinamiformes
Apodiformes
Caprimulgiformes
Piciformes
Coraciiformes
Bucerotiformes
Trogoniformes
Strigiformes
Coliiformes
Accipitriformes

Figure S1

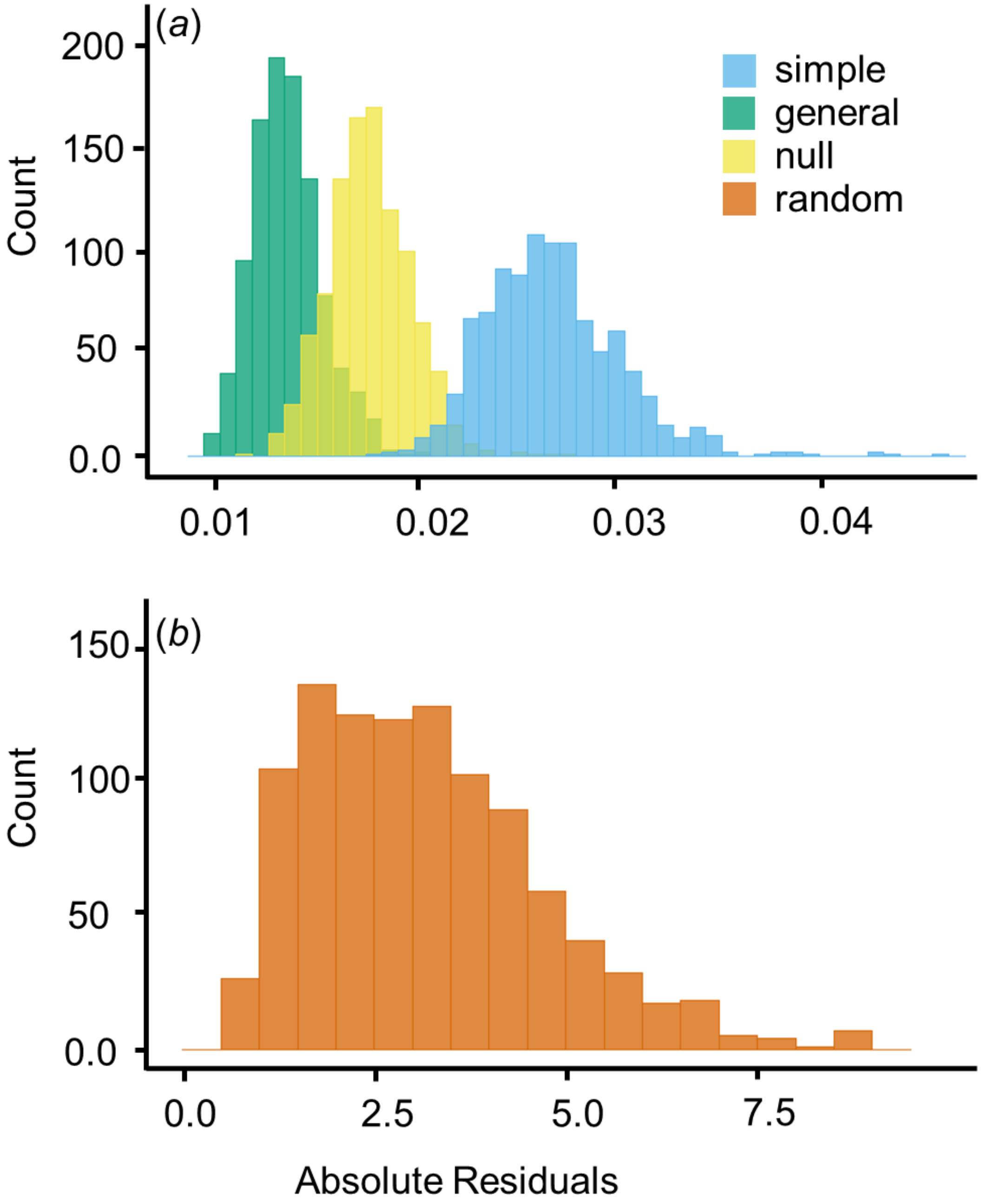
(a)
simple
general
null
random
Count
200
150
100
50
0.0
0.01
0.02
0.03
0.04
(b)
Count
150
100
50
0.0
0.0
2.5
5.0
7.5
Absolute Residuals

Figure S2

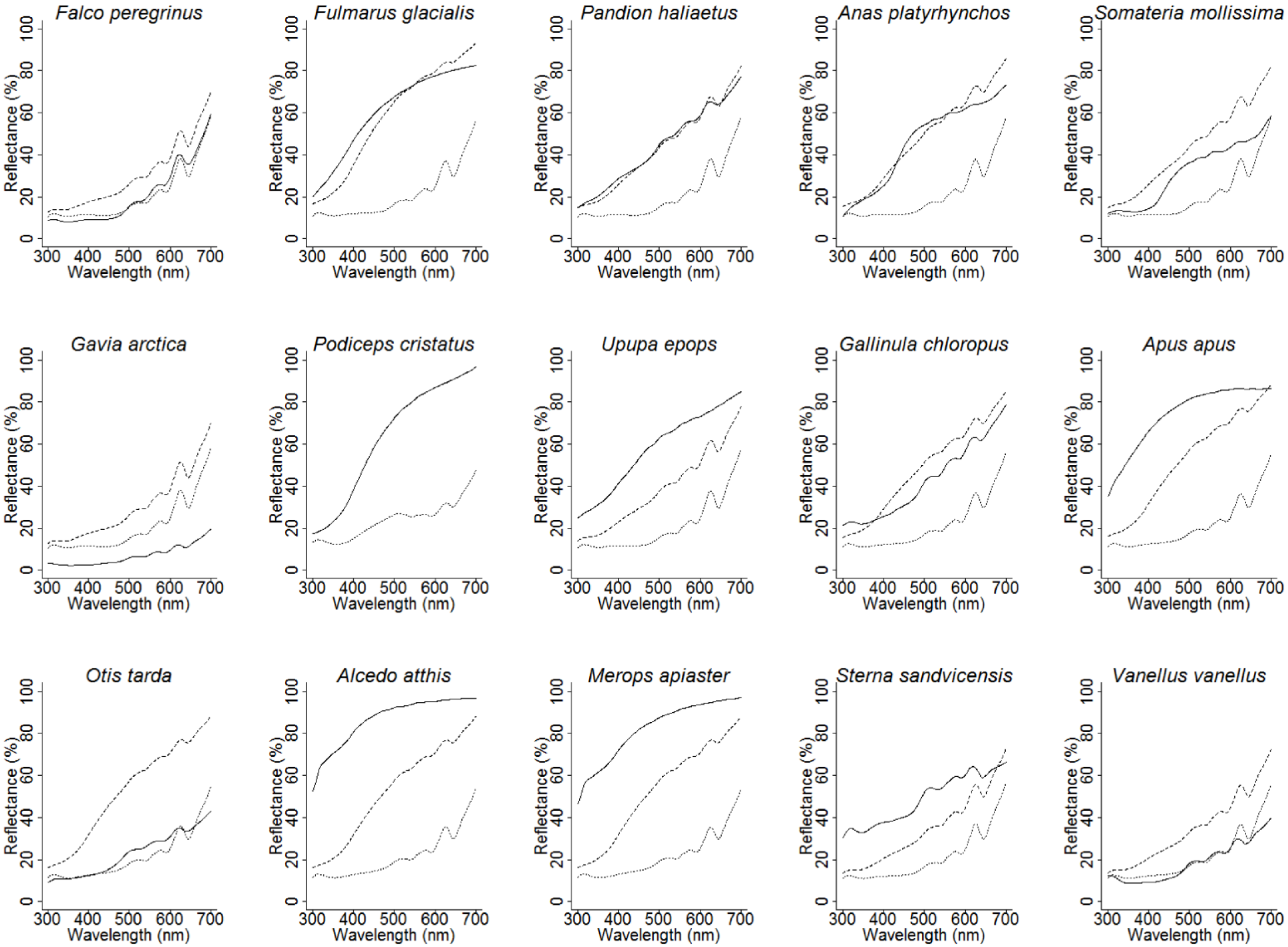
Falco peregrinus
Fulmarus glacialis
Pandion haliaetus
Anas platyrhynchos
Somateria mollissima
Gavia arctica
Podiceps cristatus
Upupa epops
Gallinula chloropus
Apus apus
Otis tarda
Alcedo atthis
Merops apiaster
Sterna sandvicensis
Vanellus vanellus
Reflectance (%)
0
20
40
60
80
100
Wavelength (nm)
300
400
500
600
700

Figure S3

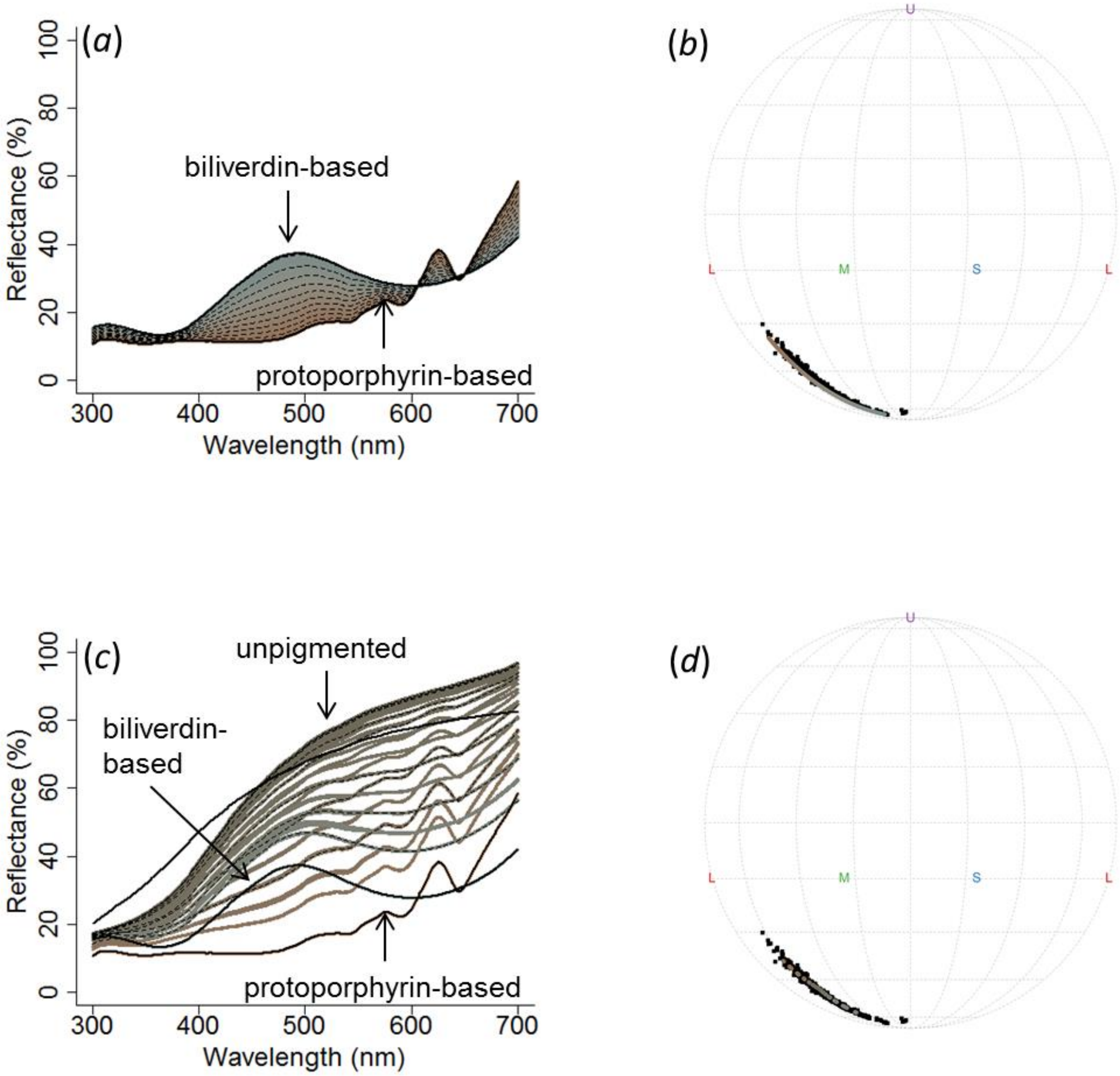
(a)
biliverdin-based
protoporphyrin-based
Reflectance (%)
0
20
40
60
80
100
300
400
500
600
700
Wavelength (nm)
(b)
U
L
M
S
L
(c)
unpigmented
biliverdin-
based
protoporphyrin-based
Reflectance (%)
0
20
40
60
80
100
300
400
500
600
700
Wavelength (nm)
(d)
U
L
M
S
L

Figure S4

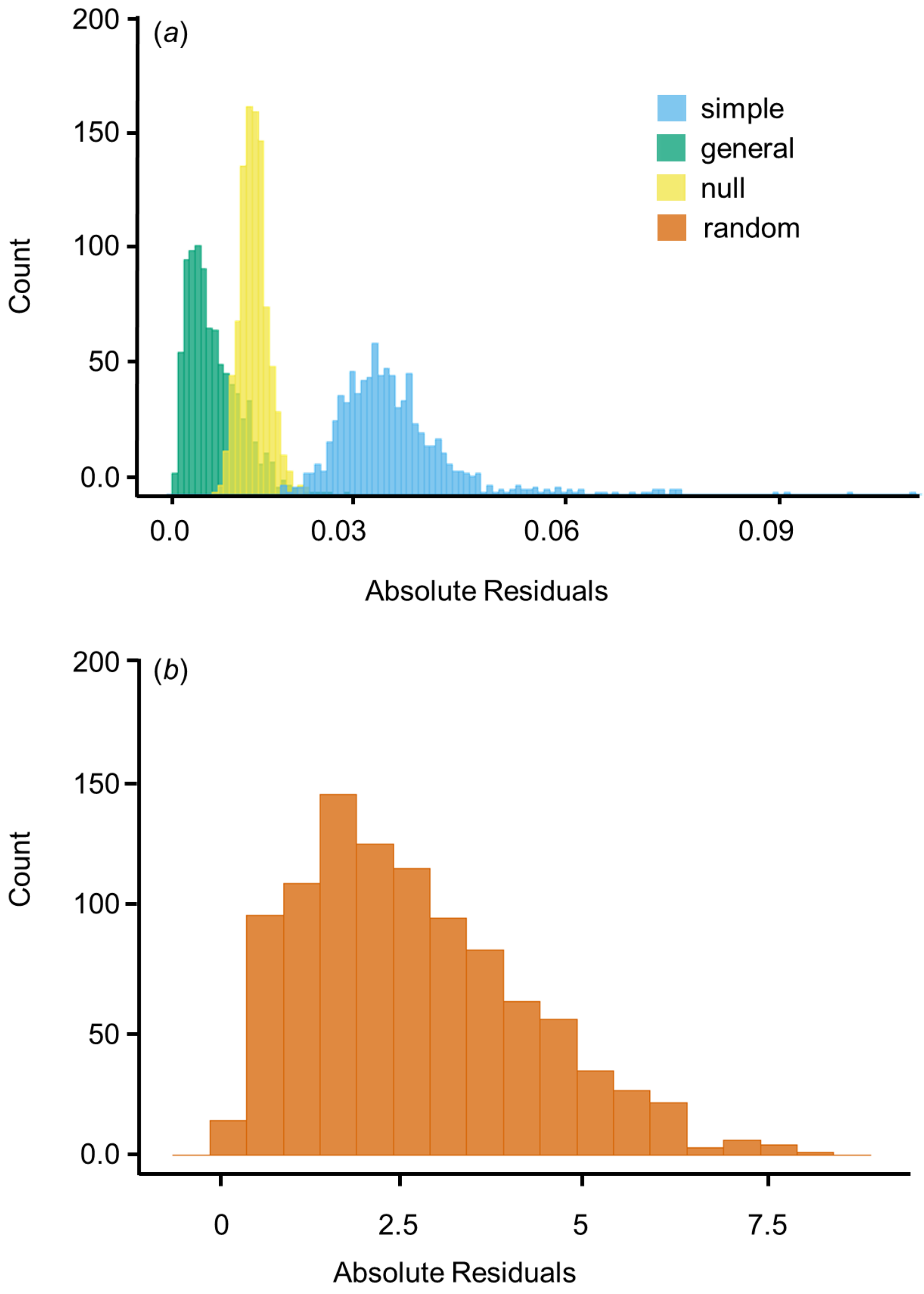
(a)
200
150
100
50
0.0
Count
simple
general
null
random
0.0
0.03
0.06
0.09
Absolute Residuals
(b)
200
150
100
50
0.0
Count
0
2.5
5
7.5
Absolute Residuals

Figure S5