doi:10.1111/evo.12855

Evolutionary shifts in the melanin-based color system of birds

Chad M. Eliason,[1,2] Matthew D. Shawkey,[3,4] and Julia A. Clarke[1]

[1] *Department of Geological Sciences and Integrated Bioscience, University of Texas at Austin, Austin, Texas 78712*

[2] *E-mail: chad_eliason@utexas.edu*

[3] *Department of Biology and Integrated Bioscience, University of Akron, Akron, Ohio, 44325*

[4] *Current address: University of Ghent, Department of Biology, Terrestrial Ecology Unit, Ledeganckstraat 35, B-9000, Ghent, Belgium*

Received August 28, 2015
Accepted December 18, 2015

Melanin pigments contained in organelles (melanosomes) impart earthy colors to feathers. Such melanin-based colors are distributed across birds and thought to be the ancestral color-producing mechanism in birds. However, we have had limited data on melanin-based color and melanosome diversity in Palaeognathae, which includes the flighted tinamous and large-bodied, flightless ratites and is the sister taxon to all other extant birds. Here, we use scanning electron microscopy and spectrophotometry to assess melanosome morphology and quantify reflected color for 19 species within this clade. We find that brown colors in ratites are uniquely associated with elongated melanosomes nearly identical in shape to those associated with black colors. Melanosome and color diversity in large-bodied ratites is limited relative to other birds (including flightless penguins) and smaller bodied basal maniraptoran dinosaur outgroups of Aves, whereas tinamous show a wider range of melanosome forms similar to neognaths. The repeated occurrence of novel melanosome forms in the nonmonophyletic ratites suggests that melanin-based color tracks changes in body size, physiology, or other life history traits associated with flight loss, but not feather morphology. We further anticipate these findings will be useful for future color reconstructions in extinct species, as variation in melanosome shape may potentially be linked to a more nuanced palette of melanin-based colors.

KEY WORDS: **ratites, scanning electron microscopy, pigments, evolutionary rates.**

Colorful feathers play diverse roles in the lives of birds. These roles range from ecological (Stettenheim 2000) to ornamental (Andersson 1994). Feather colors are caused either by light scattering from feather tissues (structural colors; Prum 2006) or light absorption by pigments (McGraw 2006a,b). Color diversity depends on external (natural, sexual selection) as well as internal factors (e.g., how a color is produced). Recent comparative studies in birds have revealed that innovations in how colors are produced can enhance the range of colors displayed (Maia et al. 2013; Thomas et al. 2014). For example, the repeated evolution of red and yellow carotenoid pigments in birds (Thomas et al. 2014) expanded the avian color palette compared to yellow-brown and black colors produced by melanin pigments.

Melanin-based coloration is the ubiquitous color-producing mechanism in birds (McGraw 2006a). There are two types of melanin pigments in feathers: eumelanin, imparting brown, gray, and black hues; and phaeomelanin, imparting red and yellow hues (McGraw 2006aa). Both types of melanin are contained in organelles called melanosomes. Eumelanin is generally packaged in rod-shaped melanosomes, and phaeomelanin is typically contained in smaller, more spherical melanosomes (Trinkaus 1948), although both pigments may be found within the same melanosome (Simon et al. 2008). This relationship between pigment type and melanosome shape has enabled the reconstruction of the colors of extinct taxa (Vinther et al. 2008; Clarke et al. 2010; Li et al. 2010, 2012).

Within birds, melanin-based coloration has been suggested as the only ancestral color-producing mechanism in feathers (Stoddard and Prum 2011), with additional ways of producing color (e.g., carotenoids) evolving later (Thomas et al. 2014). Recent analyses of color mechanisms in birds have either not sampled palaeognath birds (Stoddard and Prum 2011) or only included a

EVOLUTION
INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

limited subsample of extant diversity in the clade (Li et al. 2014; Thomas et al. 2014). Palaeognaths (60 species; Gill and Donsker 2015) comprise the sister taxon to all other living birds. They include the monophyletic, flighted tinamous, and a paraphyletic group of large, flightless species (ostriches, cassowaries, emus, kiwis, and rheas) hereafter collectively referred to as "ratites" (see Baker et al. 2014; Mitchell et al. 2014). Understanding the coloration mechanisms in both neognaths and paleognaths is crucial to reconstructing ancestral color-producing mechanisms in the avian crown clade and understanding broader patterns of color diversity in birds (e.g., see Crisp and Cook 2005). Phylogenetic relationships within palaeognaths have been contentious (Cracraft 1974; Cooper et al. 2001; Haddrath and Baker 2001; Harshman et al. 2008; Phillips et al. 2009; Baker et al. 2014; Mitchell et al. 2014), but recent analyses of molecular sequence data indicate that tinamous are nested within palaeognaths rather than sister to ratites, suggesting numerous convergent losses of flight in various ratite lineages (Phillips et al. 2009; Baker et al. 2014; Mitchell et al. 2014).

Unlike neognaths, palaeognaths appear to lack carotenoid pigments (Thomas et al. 2014) and structurally colored feathers (Prum 2006), although tinamou eggshells are structurally colored (Igic et al. 2014). Recent evidence in a limited sample of palaeognaths showed a diminished range of melanosome morphologies relative to other living birds (Neognathae), extinct birds (basal Avialae), and other maniraptoran dinosaurs (Li et al. 2014). This limited diversity may occur because palaeognaths are simply less colorful than neognaths, or because other phenotypic traits like feather structure or physiology limit melanosome diversity independent of color (Hellström et al. 2011; Li et al. 2014). Teasing apart these hypotheses requires fine-scale color data and broad sampling of melanosome morphology across a range of palaeognaths. Palaeognaths have low mass-specific basal metabolic rates (Maloney 2008), diverse mating systems, and reversed sex roles (males in all species but the ostrich do most of the incubating and parental care; Handford and Mares 1985). In addition to these life history and physiological traits, palaeognaths are generally monochromatic (Davies 2002) and have a number of derived feather traits, including open pennaceous feathers in most ratites (i.e., feathers lacking barbule hooklets that link adjacent feather barbs; McGowan 1989) and fused feather barbules in tinamou flight and contour feathers (Chandler 1916).

Here, we use UV-Vis reflectance spectrophotometry and scanning electron microscopy (SEM) to assess the melanin-based color system in palaeognaths. We hypothesized that known differences in physiology or feather morphology within palaeognaths would be associated with differences in melanosome morphology and color disparity. Specifically, we asked: (i) Is the reported pattern of limited melanosome morphospace in palaeognaths still recovered with denser taxon sampling?; (ii) Do patterns of color diversity parallel those in melanosome diversity?; (iii) Does fine-scale spectral reflectance data affect inferences on the relationship between melanosome form and color in birds?; and (iv) What is the evolutionary history of changes in the melanin-based color system of birds?

Methods

SPECIMEN SAMPLING

We selected the most divergent color patches in 19 exemplar species from nearly all extant genera in Palaeognathae (except *Nothoprocta*), thereby maximizing both phenotypic and phylogenetic distance (Fig. 1). New feather samples were collected and combined with a previous dataset (Li et al. 2014). In total, we assessed melanosome morphology in 38 palaeognath feathers (20 feathers from 11 tinamou species, 18 feathers from eight ratite species; see Fig. 1 and Table S1 for specimen numbers). To more broadly assess the relationship between melanosome shape and color across Aves, we also measured reflectance for 27/45 black (60%), 17/35 brown (49%), 23/35 gray (66%), and 6/18 "penguin-type" (33%) neognath feathers represented in the Li et al. (2014) dataset and additionally sampled three neognath species with yellow plumage (see Table S1).

MELANOSOME MEASUREMENTS

We dissected a single feather barb from each sampled feather. We then imaged feather cross-sections and measured melanosomes following previous methods (Li et al. 2010). Briefly, we embedded and cut thin (5-μm thick) longitudinal sections of feather barbs, sputter coated these sections with palladium/platinum (Cressington 208 Sputter Coater) and imaged them with a scanning electron microscope (Ziess Supra 40VP SEM). We analyzed images in ImageJ and measured the following parameters for melanosomes in each feather: melanosome diameter, melanosome length, and melanosome density (the number of melanosomes per unit area of feather in μm^2). We then calculated the aspect ratio of melanosomes (length/diameter). We observed melanosomes in all palaeognaths sampled except for a pale yellowish-white feather from the elegant-crested tinamou (*Eudromia elegans*). We did not expect to find melanosomes in this whitish feather but included it based on previous reports of melanosomes in white chicken feathers (Bohren et al. 1943; Lucas and Stettenheim 1972). We assessed repeatability among six different readers measuring the same melanosomes multiple times. To assess whether our sampling adequately represents the range of phenotypic diversity in the group, we used a rarefaction of variance technique (see Claramunt 2010 for R code) and assessed whether variation-sample size plots reached an asymptote, indicating increased sampling would not drastically improve the captured range of variation (see Supporting Information).

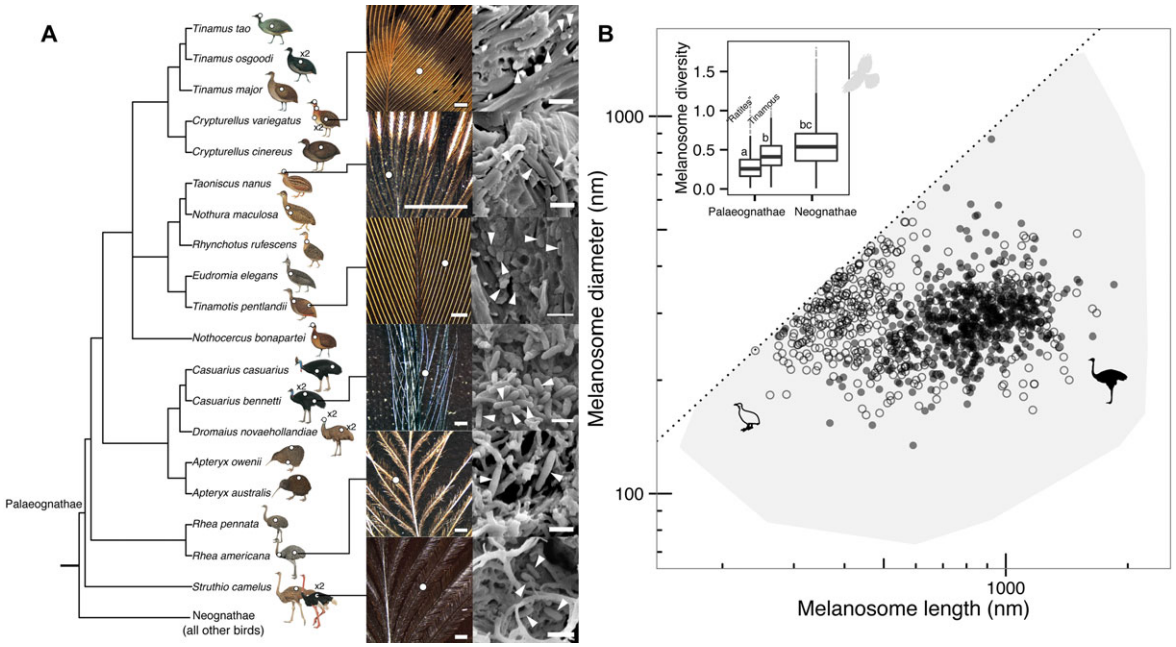

Figure 1. **Limited melanosome diversity in palaeognaths. (A) Phylogeny of sampled palaeognaths species (feather regions represented by white circles) along with representative feather (left) and SEM images (right). Feather identification, from top to bottom: variegated tinamou (*Crypturellus variagatus*) banded contour feather, dwarf tinamou (*Taoniscus nanus*) black crown feather, puna tinamou (*Tinamotis pentlandii*) yellow rump feather, dwarf cassowary (*Casuarius bennetti*) black contour feather, rhea (*Rhea spp.*) brown contour feather, and common ostrich (*Struthio camelus*) black contour feather (scale bars = 1000 um). Arrows in SEM images depict melanosomes, scale bars = 1 um. (B) Morphospace plot showing raw melanosome measurements in tinamous (open circles, $n = 475$) and ratites (closed circles, $n = 588$). Gray polygon represents extent of neognath morphospace. Insets: boxplots of Euclidean distance from group centroid (box edges: 25 and 75% quartiles, whiskers: 95% CI, horizontal line: median, points: outliers). Boxplots sharing the same letter (a, b, c) are not significantly different from one another (Tukey HSD tests; $P < 0.05$). Comparative analysis of species means also showed lower rates of melanosome shape evolution in ratites compared to other birds ($\sigma^2_{ratites} = 1.13 \times 10^{-3}$, $\sigma^2_{other\ birds} = 8.32 \times 10^{-3}$; multivariate rate test $P_{sim} = 0.001$; Adams 2014). Phylogenetic tree sources: Mitchell et al. (2014) and Bertelli and Porzecanski (2004). Illustrations reproduced with permission from the *Handbook of the Birds of the World. Vol. 1. Ostrich to Ducks*, Lynx Editions 1992 (see Supporting Information for individual species references).**

COLOR MEASUREMENT

We used a spectrophotometer (Avantes AvaSpec 2048 with xenon light source) to measure the spectral reflectance of feathers with the light source and detector nearly perpendicular to the feather surface (~8º, determined by the size of the two probes) over a wavelength range of 300–700 nm (i.e., the bird-visible spectrum; Bennett et al. 1994). For each spectrum, we took the average of 10 scans at 1 pulse per 100 ms integration time. Ratite feathers were generally uniformly colored compared to tinamous (Fig. 1A). By contrast, in tinamous, color varied continually across a feather (e.g., brown-black gradients in the banded contour feathers of *Crypturellus variegatus*) and discretely at anatomical boundaries (e.g., between yellow barb rami and black barbules in yellow rump feathers of *Tinamotis pentlandii*; see Fig. 1A). Thus, we were careful to measure spectral reflectance close to the location where we removed feather barbs for SEM.

We analyzed color in three ways: (i) using a principal component analysis (PCA) to capture the range of variation in spectral shape, (ii) using spectral shape variables including mean brightness (B2) and saturation (S5; see Montgomerie 2006) and (iii) using avian visual models for comparison with previous work on melanin-based coloration in birds (see Supplemental Methods and Results). We ran the PCA analysis in R using the prcomp package on raw, unscaled spectral data divided into 20-nm wavelength bins (Cuthill et al. 1999). Color variables were used as response variables in subsequent multiple regression analyses. To assess whether our sampling captured the range of color variation in palaeognaths, we ran rarefaction analyses on the color variables as described above (see Supporting Information).

MORPHOLOGICAL AND COLOR DIVERSITY ANALYSES

To test for differences in melanosome diversity among clades, we used two approaches. First, to test whether the overall range of melanosome forms differed, we used a randomization test. Briefly, we computed the convex hull volumes enclosing all points in

different groups. We then shuffled group identity among the points 5000 times and recalculated convex hull volumes for each group. We then computed the ratio of the two volumes and used this as a null distribution for comparison with the observed volume ratio. Second, because convex hull volumes are sensitive to outliers and can therefore overestimate the amount of disparity in a group (Wills 2001), we also calculated the Euclidean distance of each melanosome measurement from the mean morphology of the group (Wills et al. 1994). Because our dataset contained multiple measurements within feathers, as well as within species, and because we were interested in comparing morphological variation at different levels of biological organization, we fit linear-mixed models in the lme4 package with patch and species as random effects, group membership as a fixed effect, and distance to the group centroid as the response variable. We assessed significance of fixed effects in linear-mixed models using Kenward–Roger approximation in the pbkrtest package (Halekoh and Højsgaard 2014). To account for phylogenetic relationships among species, we also compared rates of melanosome shape evolution in a comparative framework using the compare.evol.rates function in the geomorph package in R (see Supporting Information).

MELANOSOME SHAPE-COLOR RELATIONSHIP

To test for a relationship between melanosome shape and feather color, we again used linear-mixed models in the lme4 package to account for replicate sampling within birds (i.e., multiple plumage patches). We fit a full model with the coefficient of variation and skewness of melanosome length and diameter, melanosome density, aspect ratio, and melanosome volume (calculated using the equation for the volume of an ellipsoid: $4/3\pi$ x length x diameter^2; Hurbain et al. 2008; Zwillinger 2011) as predictor variables and species identity as a random effect. We then used the dredge function in the R package MuMIn to select the most parsimonious model from among all possible submodels ranked by AICc value (suitable for small sample sizes). In addition to PC1 and PC2, we also ran these models with alternative color metrics (see Fig. S10). Results of linear-mixed model analyses indicated little among species variation in saturation (PC2), with most variation occurring among plumage patches. However, brightness (PC1) showed strong species effects, suggesting potential phylogenetic structure in the residual error. Therefore, to account for this, we also fit Bayesian phylogenetic-mixed models (BPMMs) using the MCMCglmm package (Hadfield and Nakagawa 2010; see Supporting Information). This is currently the only phylogenetic regression method that allows for multiple measurements per species. To compare whole reflectance spectra, we used the anosim function in the R package vegan (Clarke 1993). This function ranks similarities among all reflectance values and uses a permutation procedure to estimate difference in the number of rank similarities between versus within samples (e.g., reflectance spectra of ratites versus tinamous). A large value of the R statistic would indicate that two sets of spectra are significantly different from each other.

GENERAL STATISTICAL CONSIDERATIONS

We performed all statistical analyses in R v. 3.1.2. We transformed response variables when necessary using the boxcox function in the MASS package and used various diagnostics plots to assess the normality of model residuals.

Results

MELANOSOME DIVERSITY

Palaeognath melanosomes were significantly less variable than neognaths (Monte Carlo randomization test of convex hull volumes, $P < 0.001$, $n = 18$ palaeognath, and $n = 55$ neognath species) but were just above the cutoff for being significantly more clustered within this limited range (linear-mixed model of centroid distances, $F_{1,118.5} = 3.80$, $P = 0.053$; see Fig. 1B). By "clustered" we mean that, on average, species are not very different from the mean morphology for the group. Rates of melanosome shape evolution were significantly higher in neognaths compared to palaeognaths (see Supporting Information). Within Palaeognathae, tinamous, and ratites produced a similar range of melanosomes (Monte Carlo randomization test of convex hull volumes, $P = 0.54$), but most ratite species had similar forms (i.e., they were more clustered; $F_{1,13.2} = 20.0$, $P < 0.001$; Fig. 1B) and the rate of melanosome shape evolution was significantly lower in ratites compared to other birds (see Supporting Information, Fig. 1B). Ratite melanosomes ($n = 8$ species) were also significantly less diverse than melanosomes in another clade of flightless birds with modified feather morphologies, extant penguins ($n = 11$ species; linear-mixed model of centroid distances; $F_{1,19.5} = 37.2$, $P < 0.001$; Fig. S3). Interestingly, tinamou and neognath species showed no significant difference in how their melanosomes were spread out in morphospace ($F_{1,97.1} = 1.39$, $P = 0.24$; Fig. 1B, inset).

COLOR DIVERSITY

Avian visual model analyses indicated that palaeognaths occupied a much smaller portion of avian melanic colorspace than neognaths (Table S2, Fig. 2). The overall range of colors in ratites and tinamous did not significantly differ (Monte Carlo randomization test of convex hull volumes, $P = 0.64$), and both groups were similarly clustered within this colorspace (repeated measures ANOVA of centroid distances; $F_{1,15.0} = 0.21$, $P = 0.65$; see Fig. S4B).

MELANOSOME SHAPE-COLOR RELATIONSHIP

Melanosome shape and color were correlated in neognaths and tinamous, but in ratites elongated melanosomes associated with brown color caused decoupling between melanosome shape and

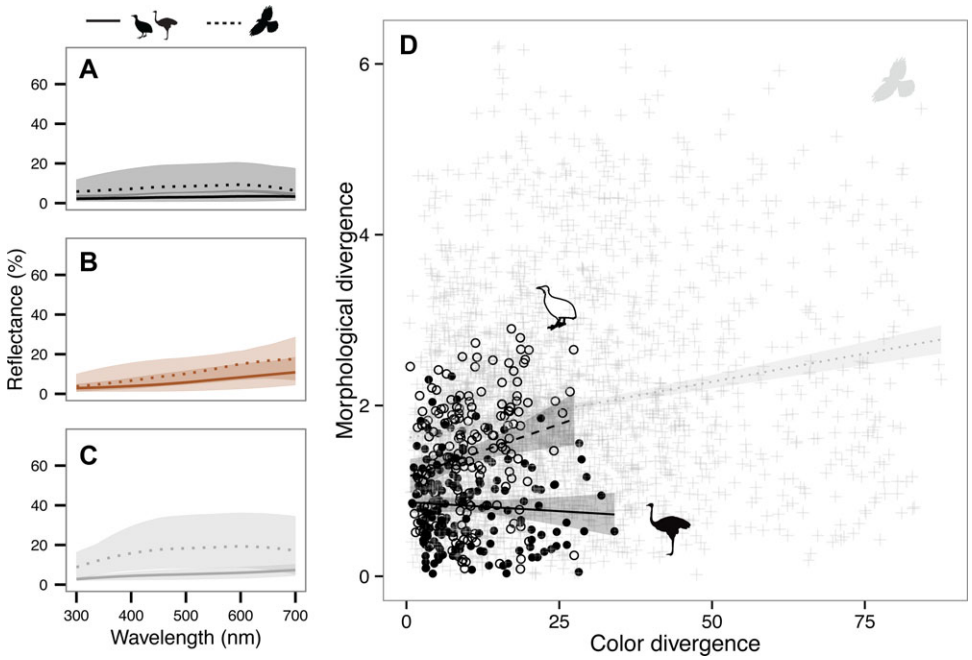

Figure 2. **Spectral and morphological diversity in birds. (A–C) Mean reflectance spectra in neognaths (dotted lines) and palaeognaths (solid lines) for black (A), brown (B), and gray feathers (C). Shaded regions are 95% confidence intervals. (D) Relationship between color divergence (Euclidean distance between reflectance values at all wavelengths between 300–700 nm) and morphological divergence (Euclidean distance between all pairs of melanosomes length and diameter measurements). Points shapes represent tinamous (open circles), ratites (filled circles), and neognaths (gray crosses). Lines are linear regression fits and 95% confidence intervals (shaded regions).**

predicted color. Melanosome morphology for all avian taxa sampled explained 46% of the variation in feather color saturation ($X^2 = 61.6$, df = 3, $P < 0.001$) and 36% (14% without accounting for within-species variation) of the variation in feather brightness ($X^2 = 25.7$, df = 5, $P < 0.001$). Brightness (PC1) increased with both melanosome length variability within a feather ($F_{1,103.9} = 10.38$, $P = 0.0017$; Fig. S9C) and melanosome density ($F_{1,104.0} = 5.46$, $P = 0.021$; Fig. S9D), and decreased with melanosome aspect ratio ($F_{1,101.1} = 4.81$, $P = 0.031$; Fig. S9A). Brightness was not significantly related to melanosome volume ($F_{1,102.6} = 3.90$, $P = 0.051$). Saturation (inversely related to PC2) decreased with melanosome aspect ratio ($F_{1,100.5} = 12.86$, $P < 0.001$; Fig. S9E), melanosome size ($F_{1,104.0} = 36.40$, $P < 0.001$; Fig. S9F), melanosome length variability within a feather ($F_{1,103.4} = 10.41$, $P = 0.0017$; Fig. S9G) and melanosome density ($F_{1,103.1} = 7.40$, $P = 0.0079$; Fig. S9H).

Analysis of all pairwise differences in mean color and melanosome morphology showed that more divergent colors (based on differences in reflectance spectra) were associated with more divergent melanosomes in tinamous (Mantel $r = 0.25$, $P = 0.011$) and neognaths ($r = 0.11$, $P = 0.014$), but not in ratites ($r = -0.08$, $P = 0.63$; Fig. 2D). This weak shape-color relationship in ratites did not affect multiple regression results, which were similar after excluding ratites, as well as with alternative color metrics (Fig. S10) and incorporating phylogeny (Fig. S11).

Within Palaeognathae, melanosomes in brown tinamou feathers were much shorter than those in black feathers (brown = 475 ± 37 nm, black = 1033 ± 63 nm; $P_{Tukey} < 0.001$; Fig. 3A,C). By contrast, melanosomes in brown ratite feathers were nearly indistinguishable from those in black feathers (brown = 830 ± 44 nm, black = 849 ± 43 nm; $P_{Tukey} > 0.05$; Fig. 3A,C). Reflectance spectra did not differ significantly between tinamous and ratites for black (analysis of similarity of reflectance values; $R = -0.10$, $P = 0.76$; Fig. 3B) or brown feathers ($R = 0.050$, $P = 0.22$; Fig. 3D), suggesting similarities in melanin pigments within melanosomes.

RATITE MELANOSOME SHAPE AND COLOR DIVERSITY COMPARED TO OTHER AMNIOTES

Both ratites and extant nonavian reptiles showed a similar pattern of limited melanosome shape diversity (Fig. 4). However, ratites had more elongated melanosomes than extant nonavian reptiles, both in black ($P_{Tukey} = 0.047$; overall model $F_{4,67.5} = 19.1$, $P < 0.001$) and brown integuments ($P_{Tukey} < 0.001$; overall model $F_{4,74.2} = 7.93$, $P < 0.001$). Melanosomes from brown ratite feathers were longer than melanosomes from brown integuments in all other extant amniote groups ($P_{Tukey} < 0.001$), but black ratite melanosomes did not differ significantly in aspect ratio from those in mammals or neognaths ($P_{Tukey} > 0.05$; Fig. 4). Comparative analyses revealed several evolutionary increases in melanosome

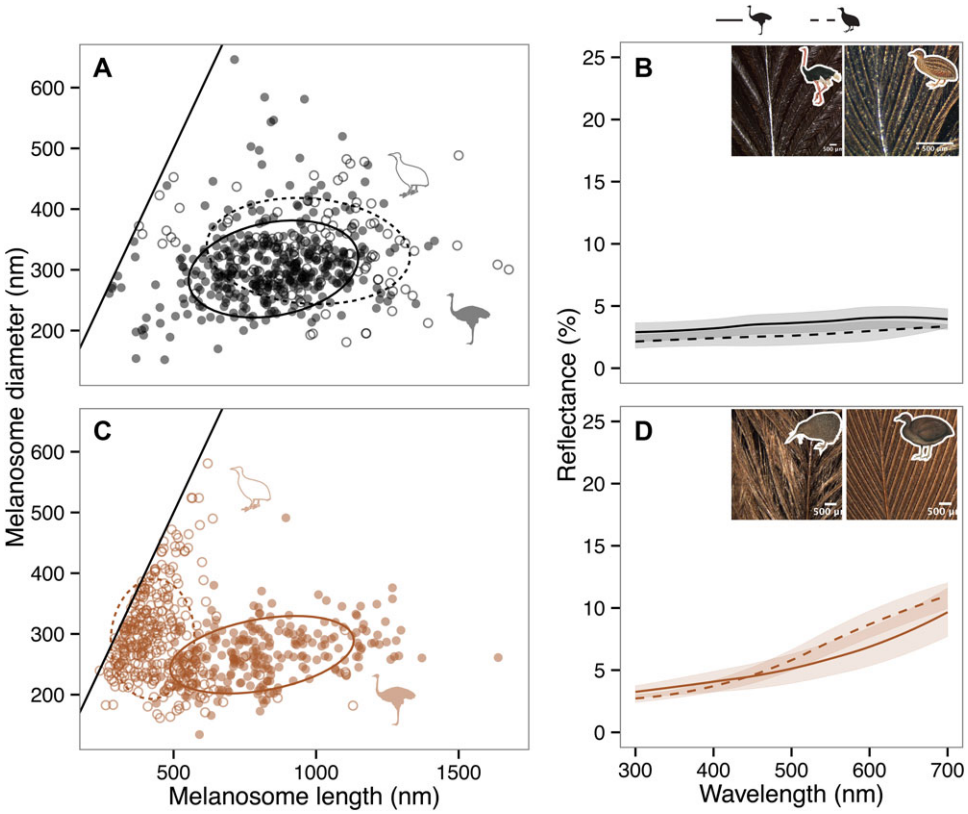

Figure 3. **Decoupled morphological and color diversity in ratites. (A, C) Plots of raw melanosome measurements from black (A) and brown palaeognaths feathers (C). Points represent tinamous (open circles) and ratites (closed circles). (B, D) Mean reflectance spectra in ratites (solid lines) and tinamous (dashed lines). Shaded regions depict ±1 standard errors. Insets show representative feather images for each group (scale bars = 500 um).**

aspect ratio associated with brown colors in ratites (Fig. S8) as well as a significant, positive relationship between melanosome aspect ratio and flightlessness (Table S3).

Discussion

Our results show that color quantitatively assessed via reflectance data can be linked to melanosome morphology across birds. Furthermore, they provide evidence for several changes in melanosome shape independent of variation in coloration in ratites and confirm previous reports of limited melanosome diversity in palaeognaths (Li et al. 2014), specifically large-bodied ratites. Melanin-based colors were also unsurprisingly less diverse in paleognaths than in neognaths. Given that faster speciation rates have been linked to dichromatism (Owens and Bennett 1999; Wagner et al. 2012) and color diversity, both within (Hugall and Stuart Fox 2012) and between species (Maia et al. 2013; but see Huang and Rabosky 2014), weaker sexual selection in ratites and tinamous may partially explain their lower species richness and muted color diversity.

Recent discoveries have indicated that shifts in the melanin-based color system are common in nature. For example, the evolution of iridescent colors is associated with uniquely long and thin melanosomes in many extant birds and extinct paravian dinosaurs (Li et al. 2012), and black colors in mammal hairs and bird feathers are associated with elliptical melanosomes compared to the more spherical forms in nonavian reptiles (Li et al. 2014). Our results reveal an additional shift in brown feathers, from spherical melanosomes found in most birds and nonavian reptiles to more elliptical melanosomes in various ratite lineages (Fig. 4, Fig. S8A). Mechanistically, variation in the proportions of eumelanin and phaeomelanin within melanosomes might explain this pattern (Fig. 3C). Melanin proportions are known to vary within feathers (McGraw 2006a), within melanocytes (Inazu and Mishima 1993) and even within melanosomes (Simon et al. 2008). Ratites thus may also show shifts in the proportions of phaemelanin and eumelanin within similarly shaped melanosomes.

Evolutionarily, the pattern in Palaeognathae could be explained by multiple convergent changes in melanosome shape and diversity in different ratite lineages or, alternatively, a single shift in the most recent common ancestor of extant palaeognaths followed by a reversal to the neognath pattern in tinamous (Scenarios 1 and 2 in Fig. 4). Ancestral state reconstructions using maximum likelihood support the multiple convergent

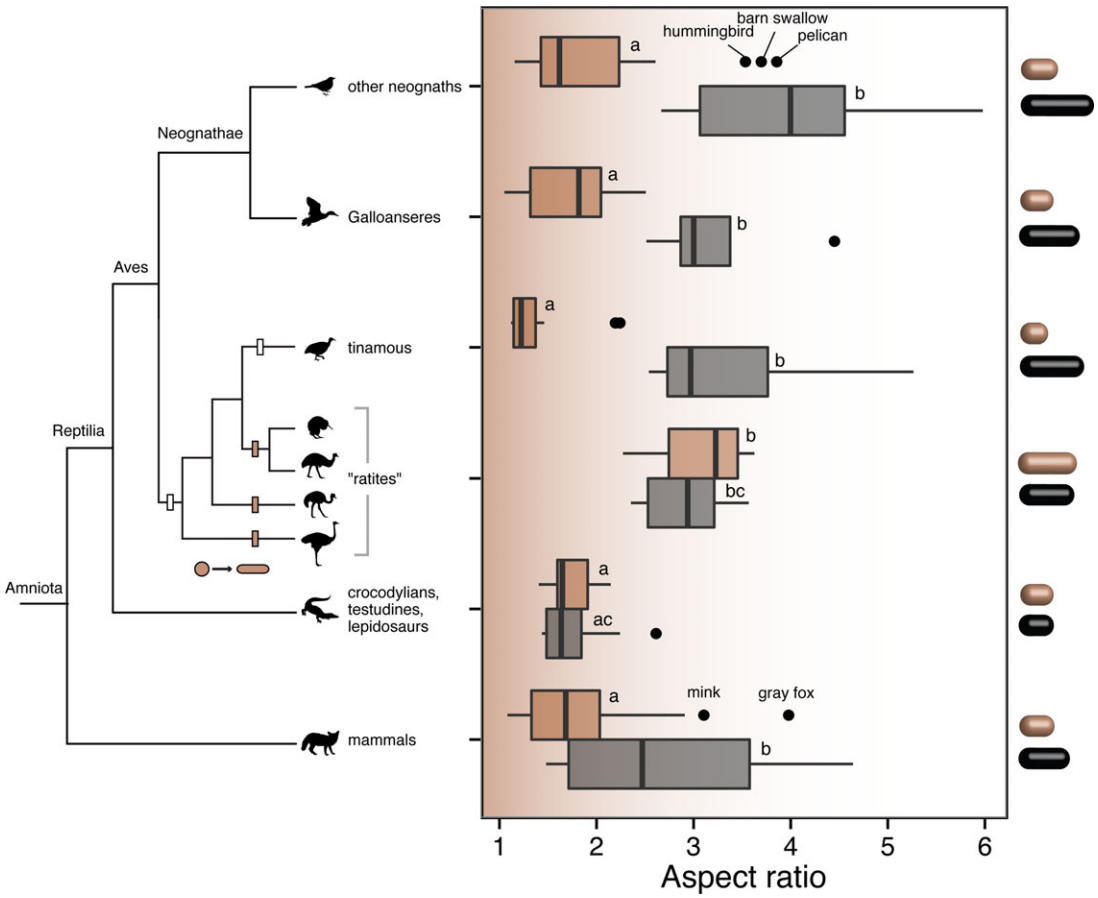

Figure 4. Evolution of a novel melanosome shape-color relationship in large, flightless "ratite" birds. Phylogeny of representative amniote groups and boxplots showing distribution of melanosome aspect ratios (length/diameter) in brown (upper boxes) and black feathers (lower boxes in each group; color online). Boxplots sharing the same letter (a, b, c) are not significantly different from one another (two-sided Tukey HSD; $P < 0.05$; black and brown colors analyzed separately). Outsets at right are mean melanosome shapes. Vertical brown boxes on branches of the phylogeny indicate estimated increases in melanosome aspect ratio in brown plumages away from the state in the most-recent common ancestor (MRCA) of palaeognaths (Scenario 1). Open boxes represent an alternative scenario of increased aspect ratio in the MRCA of palaeognaths followed by a reversal to more spherical, "neognath-like" melanosomes associated with brown feathers in tinamous (Scenario 2). Based on the limited diversity seen in crocodylians, testudines, and lepidosaurs (Li et al. 2014); the increased melanosome diversity in maniraptoran dinosaurs (Li et al. 2014); and (iii) ancestral state reconstructions of melanosome morphology associated with brown coloration (Fig. S8A), the multiple gains hypothesis (Scenario 1) is preferred. Silhouettes drawn by Francesco Architetto Rollandin, Sharon Wegner Larsen, Darren Naish, Steven Traver, Matt Martyniuk, B. Kimmel, and Rebecca Groom or modified by T. Michael Keesey were downloaded from http://phylopic.org.

gains hypothesis (Fig. 4, Fig. S8A). Recent evidence for diverse melanosome forms in extinct-feathered dinosaurs (Li et al. 2014) suggests that the capability for producing spherical melanosomes like those typically found in brown feathers of neognaths was also present in early birds. This would increase the probability that the ancestral state of melanosome shape in brown feathers at the basal node of birds was spherical, further exaggerating the observed shifts to elongated melanosomes in ratites (Fig. S8A). If the ratite condition evolved in the common ancestor of extant paleognaths along with loss of flight and nonaerodynamic changes in feather structure (loss of barbule hooklets, remiges, and rectrices), then pennaceous flight feathers, high metabolic rates associated with flight (Walsberg 1983), and a neognath-type color system must have all reevolved in tinamous (Fig. 4). Whether a scenario involving multiple convergent gains of the novel ratite condition associated with flight loss or a single basal gain and subsequent reversal in tinamous associated with flight regain is correct will ultimately depend on gathering new fossil evidence and resolution of the relationships among extant and extinct palaeognaths. In either case, the significant evolutionary correlation between melanosome shape in brown feathers and flightlessness in palaeognaths (Table S3, Fig. S8) suggests that the melanin-based color system and morphological, physiological, or life history traits associated with flight capability may be proximately linked.

Whether shifts in integumentary structure and physiology cause, or are linked to, changes in the melanin-based color system in amniotes has been unclear. Evolutionary shifts in feather morphology or plumage complexity might reasonably be expected to accompany changes in melanosome diversity because (i) processes that occur within feathers determine the uptake and production of different melanin pigments and melanosome shapes (Lucas and Stettenheim 1972; Lin et al. 2013) and (ii) complex morphological structures like feathers have more "degrees of freedom" to vary (Prum and Dyck 2003; Wagner et al. 2007), and therefore greater potential for changes in melanosome shape and color among feather parts or plumage regions. The origins of some novel keratinous structures in amniotes (e.g., pinnate feathers in birds and hairs in mammals) are concomitant with increases in melanosome diversity (Li et al. 2014), while other shifts are not. For example, the shift from beta keratin scales to filaments in Archosauria and the origin of novel beta keratins (Greenwold and Sawyer 2011) in that clade are not linked to changes in melanosome diversity (Li et al. 2014). Similarly, extant penguins have uniquely large and round melanosomes relative to extinct stem penguins (as well as most other neognaths) despite similar feather morphologies (Clarke et al. 2010), suggesting that feather morphology and melanosome diversity may be decoupled.

Within Palaeognathae, the evolution of novel feather microstructures in tinamous (fused feather barbules; Chandler 1916) was not associated with a change in melanosome diversity relative to neognaths (Fig. 1B). Flightless ratites share some unique plumage attributes (Chandler 1916; McGowan 1989; Davies 2002) along with less diverse melanosomes relative to tinamous and neognaths (Fig. 4). However, at the microstructural level, ratite feathers are not particularly similar, differing in barb density, barb diameter, barb length, barbule shape (e.g., flat in ostriches versus cylindrical in rheas and cassowaries), and how barbules are distributed along the length of a barb (Chandler 1916). Variable feather microstructure despite limited melanosome diversity in ratites (Fig. 1B) suggests that shifts in melanosome morphology are not associated with changes in feather microstructure. Another group with similarly uniform plumages (lacking feather apteria) and modified feathers, penguins, shows significantly more melanosome diversity than ratites (Fig. S3), suggesting that shifts in feather microstructure and reduced plumage complexity are not the main drivers of melanosome diversity in birds. While variability in ratite feathers is consistent with the expected effects of relaxed selection (Lahti et al. 2009), for example, on aerodynamic properties of feathers, their strikingly similar melanosome morphologies are not.

Shifts in melanosome diversity might be linked to changes in physiology (Li et al. 2014). The melanocortin system has long been known to have pleiotropic effects on melanin-based coloration, as well as physiology and behavior (reviewed in Ducrest et al. 2008). For example, agouti signaling protein (ASIP) is involved in the switch from eumelanin to phaeomelanin production within melanocytes and also plays roles in fat storage and appetite regulation (Forbes et al. 2001; Ducrest et al. 2008). Consistent with the physiology hypothesis, flightless ratites known to have the lowest mass-specific basal metabolic rates in birds (Maloney 2008) also have the lowest melanosome diversity, whereas flighted tinamous with higher basal metabolic rates than ratites (Withers et al. 1987) do not differ from neognaths in melanosome diversity (Fig. 1B). Outgroup reptiles (crocodiles, turtles, lizards) with low metabolic rates (Makarieva et al. 2008) also show little difference in melanosome shape between brown and black integuments, even though their melanosomes are overall less elongated than ratites (Fig. 4). Limited melanosome diversity in ratites suggests developmental constraint, stabilizing selection, or perhaps correlated response to selection on other traits related to flight loss (e.g., body size).

The discovery that fossilized remains were not bacteria but melanosomes (Vinther et al. 2008) unlocked the capability to infer melanin-based color, including iridescence, in deep time (e.g., Clarke et al. 2010; Vinther et al. 2010; Li et al. 2010; Zhang et al., 2010; Carney et al. 2012; Li et al. 2012). Previous work on melanin-based color reconstructions has relied on qualitative color categories. However, color is a multidimensional trait varying along multiple color axes (Kemp et al. 2015). Our incorporation of quantitative reflectance data revealed associations between melanosome morphology and different aspects of coloration (brightness and saturation) that could be used to predict subtle variations in the integumentary colors of extinct taxa. Larger particles scatter light more strongly than small particles (Van De Hulst 1957) and should therefore be associated with lighter gray or yellow colors (Murphy 2001). In addition, higher melanin concentrations in feathers (denser melanosomes) should theoretically absorb more light and produce both less bright (Hecht and Zajac 2002) and less saturated colors (Andersson and Prager 2006). The observed positive trend between brightness and melanosome volume (Fig. S9B) and negative relationship between melanosome density and saturation (Figs. S9H) are consistent with these optical predictions. However, the reason for the relationship between melanosome length variability and color is unclear. One possibility is that having multiple forms of melanin within the same feather may cause a "blending" of respective reflectance curves, effectively flattening out the overall reflectance curve (i.e., lowering saturation). The positive relationship between melanosome density and brightness is also puzzling, although some studies have shown this pattern for certain forms of melanin (Bashkatov et al. 2000) or in cases where melanosomes form ordered layers (Maia et al. 2011). Because the observed density-brightness relationship was marginally nonsignificant after incorporating

phylogeny (Fig. S11A) and previous work did not find a significant relationship using discrete color variables (Li et al. 2010), further research on assessing the distribution of melanosomes within feathers for a single species is needed to clarify whether and how melanosome density might relate to color in a comparative framework. Overall, melanosome aspect ratio and volume were by far the most heavily weighted variable in color estimation (Fig. S10). These parameters are easily assessable in fossilized melanosomes and, with additional sampling, may be able to be linked to subtle differences in tonalities of melanin-based colors in birds (e.g., between yellow and reddish-brown colors; see Fig. 1A).

ACKNOWLEDGMENTS

We thank Jeremiah Trimble (Harvard Museum of Comparative Zoology) as well as and Pepijn Kamminga and Rebecca Desjardins (Rijksmuseum van Natuurlijke Histoire, Leiden) for their help with locating and sampling specimens. We thank Felician Moran, Jessica Valdez, Victor Gonzalez, Kevin Toth, Helen Vo, and Reynaldo Cabrara Jr. at The University of Texas at Austin for their valuable assistance with primary data collection. We thank members of the Clarke and Shawkey labs for comments on earlier versions of the manuscript. This work was funded by NSF grants EAR-1355292 and EAR-1251922 to J.A.C.; grants NSFEAR 1251895, HFSP RGY-0083, and AFOSRFA9550-13-0222 to M.D.S. also supported this research.

DATA ARCHIVING

The doi for our data is http://datadryad.org/resource/doi:10.5061/dryad.p56p6.

The authors declare no competing financial interests.

LITERATURE CITED

Adams, D. C. 2014. Quantifying and comparing phylogenetic evolutionary rates for shape and other high-dimensional phenotypic data. Syst. Biol. 63:166–177.

Andersson, M. 1994. Sexual selection. Princeton Univ. Press, Princeton, NJ.

Andersson, S., and M. Prager. 2006. Quantifying colors. Pp. 41–89 *in* G. E. Hill and K. J. McGraw, eds. Bird coloration, Vol. 1. Harvard Univ. Press, Cambridge, Massachusetts.

Baker, A. J., O. Haddrath, J. D. McPherson, and A. Cloutier. 2014. Genomic support for a moa-tinamou clade and adaptive morphological convergence in flightless ratites. Mol. Biol. Evol. 31:1686–1696.

Bashkatov, A. N., E. A. Genina, I. K. Vyacheslav, M. M. Stolnitz, T. A. Bashkatova, O. V. Novikova, A. Y. Peshkova, and V. V. Tuchin. 2000. Optical properties of melanin in the skin and skinlike phantoms. Proc. SPIE 4162:219–226.

Bennett, A. T. D., I. C. Cuthill, and K. J. Norris. 1994. Sexual selection and the mismeasure of color. Am. Nat. 144:848–860.

Bertelli, S., and A. L. Porzecanski. 2004. Tinamou (Tinamidae) systematics: a preliminary combined analysis of morphology and molecules. Ornitol. Neotrop. 15:93–99.

Bohren, B. B., R. M. Conrad, and D. C. Warren. 1943. A chemical and histological study of the feather pigments of the domestic fowl. Am. Nat. 77:481–518.

Carney, R. M., J. Vinther, M. D. Shawkey, L. D'Alba, and J. Ackermann. 2012. New evidence on the colour and nature of the isolated Archaeopteryx feather. Nat. Commun. 3:1–6.

Chandler, A. C. 1916. A study of the structure of feathers: with reference to their taxonomic significance. California Univ. Press, California.

Claramunt, S. 2010. Discovering exceptional diversifications at continental scales: the case of the endemic families of Neotropical suboscine passerines. Evolution 64:2004–2019.

Clarke, J. A., D. T. Ksepka, R. Salas-Gismondi, A. J. Altamirano, M. D. Shawkey, L. D'Alba, J. Vinther, T. J. DeVries, and P. Baby. 2010. Fossil evidence for evolution of the shape and color of penguin feathers. Science 330:954–957.

Clarke, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18:117–117.

Cooper, A., C. Lalueza-Fox, S. Anderson, A. Rambaut, J. Austin, and R. Ward. 2001. Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution. Nature 409:704–707.

Cracraft, J. 1974. Phylogeny and evolution of the ratite birds. Ibis 116:494–521.

Crisp, M., and L. Cook. 2005. Do early branching lineages signify ancestral traits? Trends Ecol. Evol. 20:122–128.

Cuthill, I., A. T. D. Bennett, J. Partridge, and E. Maier. 1999. Plumage reflectance and the objective assessment of avian sexual dichromatism. Am. Nat. 153:183–200.

Davies, S. J. 2002. Ratites and tinamous. Oxford Univ. Press, Oxford.

del Hoyo, J., Elliot, A. and J. Carbot. 1992. Handbook of the Birds of the World: Ostrich to Ducks. Handbook of the Birds of the World, vol. 1. Lynx Editions, Barcelona.

Ducrest, A.-L., L. Keller, and A. Roulin. 2008. Pleiotropy in the melanocortin system, coloration and behavioural syndromes. Trends Ecol. Evol. 23:502–510.

Forbes, S., S. Bui, B. R. Robinson, U. Hochgeschwender, and M. B. Brennan. 2001. Integrated control of appetite and fat metabolism by the leptin-proopiomelanocortin pathway. Proc. Natl. Acad. Sci. 98:4233–4237.

Gill, F., and D. Donsker. 2015. IOC World Bird List (v. 5.3). doi: 10.14344/IOC.ML.5.3.

Greenwold, M. J., and R. H. Sawyer. 2011. Linking the molecular evolution of avian beta (β) keratins to the evolution of feathers. J. Exp. Zool. B Mol. Dev. Evol. 316:609–616.

Haddrath, O., and A. J. Baker. 2001. Complete mitochondrial DNA geonome sequences of extinct birds: ratite phylogenetics and the vicariance biogeography hypothesis. Proc. R. Soc. B 268:939–945.

Hadfield, J. D., and S. Nakagawa. 2010. General quantitative genetic methods for comparative biology: phylogenies, taxonomies and multi-trait models for continuous and categorical characters. J. Evol. Biol. 23:494–508.

Halekoh, U., and S. Højsgaard. 2014. A Kenward-Roger approximation and parametric bootstrap methods for tests in linear mixed models—the R package pbkrtest. J Stat Software 59:1–32.

Handford, P., and M. A. Mares. 1985. The mating systems of ratites and tinamous–an evolutionary perspective. Biol. J. Linn. Soc. 25:77–104.

Harshman, J., E. L. Braun, M. J. Braun, C. J. Huddleston, R. C. K. Bowie, J. L. Chojnowski, S. J. Hackett, K.-L. Han, R. T. Kimball, Ben D. Marks et al. 2008. Phylogenomic evidence for multiple losses of flight in ratite birds. Proc. Natl. Acad. Sci. 105:13462–13467.

Hecht, E., and A. Zajac. 2002. Optics. 4th ed. Addison Wesley, San Francisco, CA.

Hellström, A. R., B. Watt, S. S. Fard, D. Tenza, P. Mannström, K. Narfström, B. Ekesten, S. Ito, K. Wakamatsu, J. Larsson et al. 2011. Inactivation of Pmel alters melanosome shape but has only a subtle effect on visible pigmentation. PLoS Genet. 7:e1002285–e1002285.

Huang, H., and D. L. Rabosky. 2014. Sexual selection and diversification: reexamining the correlation between dichromatism and speciation rate in birds. Am. Nat. 184:E101–E114.

Hugall, A. F., and D. Stuart Fox. 2012. Accelerated speciation in colour-polymorphic birds. Nature 485:631–634.

Hurbain, I., W. J. Geerts, T. Boudier, S. Marco, A. J. Verkleij, M. S. Marks, and G. Raposo. 2008. Electron tomography of early melanosomes: implications for melanogenesis and the generation of fibrillar amyloid sheets. Proc. Natl. Acad. Sci. 105:19726–19731.

Igic, B., D. Fecheyr-Lippens, M. Xiao, A. Chan, D. Hanley, P. R. L. Brennan, T. Grim, G. I. N. Waterhouse, M. E. Hauber, and M. D. Shawkey. 2014. A nanostructural basis for gloss of avian eggshells. J. R. Soc. Interface 12:20141210–20141210.

Inazu, M., and Y. Mishima. 1993. Detection of eumelanogenic and pheomelanogenic melanosomes in the same normal human melanocyte. J. Invest. Dermatol. 100:172S–175S.

Kemp, D. J., M. E. Herberstein, L. J. Fleishman, J. A. Endler, A. T. D. Bennett, A. G. Dyer, N. S. Hart, J. Marshall, and M. J. Whiting. 2015. An integrative framework for the appraisal of coloration in nature. Am. Nat. 185:705–724.

Lahti, D. C., N. A. Johnson, B. C. Ajie, S. P. Otto, A. P. Hendry, D. T. Blumstein, R. G. Coss, K. Donohue, and S. A. Foster. 2009. Relaxed selection in the wild. Trends Ecol. Evol. 24:487–496.

Li, Q., J. A. Clarke, K.-Q. Gao, C.-F. Zhou, Q. Meng, D. Li, L. D'Alba, and M. D. Shawkey. 2014. Melanosome evolution indicates a key physiological shift within feathered dinosaurs. Nature 507:350–353.

Li, Q., K.-Q. Gao, J. Vinther, M. D. Shawkey, J. A. Clarke, L. D'Alba, Q. Meng, D. E. G. Briggs, and R. O. Prum. 2010. Plumage color patterns of an extinct dinosaur. Science 327:1369–1372.

Li, Q., K.-Q. Gao, Q. Meng, J. A. Clarke, M. D. Shawkey, L. D'Alba, R. Pei, M. Ellison, M. A. Norell, and J. Vinther. 2012. Reconstruction of microraptor and the evolution of iridescent plumage. Science 335:1215–1219.

Lin, S. J., J. Foley, T. X. Jiang, C. Y. Yeh, P. Wu, A. Foley, C. M. Yen, Y. C. Huang, H. C. Cheng, C. F. Chen et al. Chuong. 2013. Topology of feather melanocyte progenitor niche allows complex pigment patterns to emerge. Science 340:1442–1445.

Lucas, A. M., and P. R. Stettenheim. 1972. Growth of follicles and feathers. Color of feathers and integument. Pp. 341–419 *in* Avian anatomy-integument. U.S. Government Printing Office, Washington, D.C.

Maia, R., D. R. Rubenstein, and M. D. Shawkey. 2013. Key ornamental innovations facilitate diversification in an avian radiation. Proc. Natl. Acad. Sci. 110:10687–10692.

Maia, R., L. D'Alba, and M. D. Shawkey. 2011. What makes a feather shine? A nanostructural basis for glossy black colours in feathers. Proc. R. Soc. B 278:1973–1980.

Makarieva, A. M., V. G. Gorshkov, B.-L. Li, S. L. Chown, P. B. Reich, and V. M. Gavrilov. 2008. Mean mass-specific metabolic rates are strikingly similar across life's major domains: evidence for life's metabolic optimum. Proc. Natl. Acad. Sci. 105:16994–16999.

Maloney, S. K. 2008. Thermoregulation in ratites: a review. Aust. J. Exp. Agr. 48:1293–1301.

McGowan, C. 1989. Feather structure in flightless birds and its bearing on the question of the origin of feathers. J. Zool. London 218:537–547.

McGraw, K. J. 2006a. Mechanics of melanin-based coloration: mechanisms and measurements. Pp. 243–294 *in* G. E. Hill and K. J. McGraw, eds. Bird coloration, vol. 1. Harvard Univ. Press, Cambridge, MA.

——. 2006b. Mechanics of carotenoid-based coloration. Pp. 177–242 *in* K. J. McGraw and G. E. hill, eds. Bird coloration, vol. 1. Harvard Univ. Press, Cambridge, MA.

Mitchell, K. J., B. Llamas, J. Soubrier, N. J. Rawlence, T. H. Worthy, J. Wood, M. S. Y. Lee, and A. Cooper. 2014. Ancient DNA reveals elephant birds and kiwi are sister taxa and clarifies ratite bird evolution. Science 344:898–900.

Montgomerie, R. 2006. Analyzing colors. Pp. 90–147 *in* K. J. McGraw and G. E. hill, eds. Bird coloration Vol. I. Harvard Univ. Press, Cambridge, MA.

Murphy, J. 2001. Additives for plastics handbook. Elsevier, Oxford, UK.

Owens, I., and P. M. Bennett. 1999. Species richness among birds: body size, life history, sexual selection or ecology? Proc. R. Soc. B 266:933–939.

Phillips, M. J., G. C. Gibb, E. A. Crimp, and D. Penny. 2009. Tinamous and moa flock together: mitochondrial genome sequence analysis reveals independent losses of flight among ratites. Syst. Biol. 59:90–107.

Prum, R. O. 2006. Anatomy, physics, and evolution of structural colors. Pp. 295–353 *in* K. J. McGraw and G. E. hill, eds. Bird coloration, Vol. I. Harvard Univ. Press, Cambridge, MA.

Prum, R. O., and J. Dyck. 2003. A hierarchical model of plumage: morphology, development, and evolution. J. Exp. Zool. B Mol. Dev. Evol. 298:73–90.

Simon, J. D., L. Hong, and D. N. Peles. 2008. Insights into melanosomes and melanin from some interesting spatial and temporal properties. J. Phys. Chem. B 112:13201–13217.

Stettenheim, P. R. 2000. The integumentary morphology of modern birds—an overview. Am. Zool. 40:461–477.

Stoddard, M. C., and R. O. Prum. 2011. How colorful are birds? Evolution of the avian plumage color gamut. Behav. Ecol. 22:1042–1052.

Thomas, D. B., K. J. McGraw, M. W. Butler, M. T. Carrano, O. Madden, and H. F. James. 2014. Ancient origins and multiple appearances of carotenoid-pigmented feathers in birds. Proc. R. Soc. B 281:20140806–20140806.

Trinkaus, J. P. 1948. Factors concerned in the response of melanoblasts to estrogen in the brown leghorn fowl. J. Exp. Zool. 109:135–169.

Van De Hulst, H. C. 1957. Light scattering by small particles. Dover Publications, Mineola, NY.

Vinther, J., D. Briggs, R. O. Prum, and V. Saranathan. 2008. The colour of fossil feathers. Biol. Lett. 4:522.

Vinther, J., D. E. G. Briggs, J. Clarke, G. Mayr, and R. O. Prum. 2010. Structural coloration in a fossil feather. Biol. Lett. 6:128–131.

Walsberg, G. E. 1983. Avian ecological energetics. Pp. 161–220 *in* D. S. Farner, J. R. King, and K. C. Parks, eds. Avian biology, Vol. 7. Academic Press, Pittsburgh, PA.

Wagner, C. E., L. J. Harmon, and O. Seehausen. 2012. Ecological opportunity and sexual selection together predict adaptive radiation. Nature 487:366–369.

Wagner, G. P., M. Pavlicev, and J. M. Cheverud. 2007. The road to modularity. Nat. Rev. Genet. 8:921–931.

Wills, M. A., D. Briggs, and R. A. Fortey. 1994. Disparity as an evolutionary index—a comparison of cambrian and recent arthropods. Paleobiology 20:93–130.

Wills, M. A. 2001. Morphological disparity: a primer. Pp. 55–104 *in* J. M. Adrain, G. D. Edgecombe, and B. S. Lieberman, eds. Fossils, phylogeny, and form: an analytical approach. Springer: New York, NY.

Withers, P. C., R. B. Forbes, and M. S. Hedrick. 1987. Metabolic, water and thermal relations of the Chilean tinamou. The Condor 89:424–426.

Zhang, Fucheng, Stuart L. Kearns, Patrick J. Orr, Michael J. Benton, Zhonghe Zhou, Diane Johnson, Xing Xu, and Xiaolin Wang. 2010. Fossilized melanosomes and the colour of Cretaceous dinosaurs and birds. Nature 463:1075–1078.

Zwillinger, D. (ed). 2011. CRC standard mathematical tables and formulae. 32nd ed. CRC Press. Boca Raton, FL.

Associate Editor: R. Brumfield
Handling Editor: J. Conner

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Species and specimen ID numbers for sampled palaeoagnath and neognaths feathers.
Table S2. Melanin-based color diversity in birds.
Table S3. Phylogenetic linear models for the relationship between flightlessness and melanosome morphology.
Figure S1. Distribution of melanosomes within feathers in five Palaeognath clades.
Figure S2. Repeatability of melanosome measurements between readers.
Figure S3. Melanosome diversity in ratites and penguins.
Figure S4. Melanin-based colors variation in palaeognaths.
Figure S5. Rarefaction of variance for morphometric traits melanosome aspect ratio (A), melanosome volume (B) and melanosome density within feathers (C).
Figure S6. Rarefaction of variance for color variables brightness (PC1, A) and saturation (PC2, B).
Figure S7. Rarefaction of variance for avian tetrahedral colorspace variables.
Figure S8. Ancestral state reconstructions of melanosome morphology in birds.
Figure S9. Linear-mixed model results for the relationship between feather color and melanosome morphology in birds.
Figure S10. Comparison of linear-mixed model results for various response variables.
Figure S11. Comparison of regression slopes estimated with different analytical methods.