The Journal of Experimental Biology 215, 1272-1277

doi:10.1242/jeb.064907

RESEARCH ARTICLE

Relative contributions of pigments and biophotonic nanostructures to natural color production: a case study in budgerigar (*Melopsittacus undulatus*) feathers

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Accepted 13 December 2011

SUMMARY

Understanding the mechanistic bases of natural color diversity can provide insight into its evolution and inspiration for biomimetic optical structures. Metazoans can be colored by absorption of light from pigments or by scattering of light from biophotonic nanostructures, and these mechanisms have largely been treated as distinct. However, the interactions between them have rarely been examined. Captive breeding of budgerigars (Aves, Psittacidae, *Melopsittacus undulatus*) has produced a wide variety of color morphs spanning the majority of the spectrum visible to birds, including the ultraviolet, and thus they have been used as examples of hypothesized structure–pigment interactions. However, empirical data testing these interactions in this excellent model system are lacking. Here we used ultraviolet–visible spectrometry, light and electron microscopy, pigment extraction experiments and optical modeling to examine the physical bases of color production in seven budgerigar morphs, including grey and chromatic (purple to yellow) colors. Feathers from all morphs contained quasi-ordered air–keratin 'spongy layer' matrices, but these were highly reduced and irregular in grey and yellow feathers. Similarly, all feathers but yellow and grey had a layer of melanin-containing melanosomes basal to the spongy layer. The presence of melanosomes likely increases color saturation produced by spongy layers whereas their absence may allow increased expression of yellow colors. Finally, extraction of yellow pigments caused some degree of color change in all feathers except purple and grey, suggesting that their presence and contribution to color production is more widespread than previously thought. These data illustrate how interactions between structures and pigments can increase the range of colors attainable in birds and potentially in synthetic systems.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/8/1272/DC1

Key words: structural color, psittacofulvin.

INTRODUCTION

Over evolutionary time, metazoans, and birds in particular, have occupied large portions of available color gamut, in part because of the diversity of color production mechanisms available to them (Stoddard and Prum, 2011). Coloration can result from selective light absorption by pigments deposited in feathers. Although more than five classes of pigments have been found in bird feathers, those most commonly present are melanins and carotenoids (McGraw, 2006). Melanins, found within membrane-bound organelles (melanosomes), can produce colors ranging from black to reddish browns and pale oranges (McGraw, 2006). Carotenoid pigments are acquired by birds through their diet (Goodwin, 1984) and are responsible for most of the bright red, orange and yellow colors (Brush, 1978). A second mechanism of color production is caused by the interaction between incident light and nano-scale reflective tissues (structural coloration) of feather barbs and barbules. Colors produced in this manner include the blue, violet, ultraviolet (UV) and iridescent parts of plumage (Auber, 1957; Dyck, 1976).

It has long been recognized that structural and pigment-based colors are not mutually exclusive and can interact with one another to attain colors not possible by either mechanism alone (Dyck, 1971a; Prum et al., 1999a). For example, the combination of structural blue colors with yellow colors caused by pigments is thought to give rise to most green plumage colors (Auber, 1957; Fox, 1976) (but see Prum et al., 1998). Surprisingly, however, with the exception of a few studies (Dyck, 1971a; Shawkey and Hill, 2005; Shawkey and Hill, 2006), the interaction between pigment and structure has not been examined in detail. Understanding these interactions is crucial because different mechanisms of plumage coloration vary in, for example, their developmental cost and thus may convey different information to conspecifics. Furthermore, they may provide inspiration for new materials with novel optical properties.

As a result of over 150 years of captive breeding, the color diversity of budgerigars [Aves, Psittacidae, *Melopsittacus undulatus* (Shaw 1805); Fig. 1A] now far exceeds that of their green wild ancestors (Taylor and Warner, 1986). Color morphs include achromatic whites and greys as well as chromatic colors ranging from purple to yellow (World Budgerigar Organisation, www.world-budgerigar.org/). These latter colors are thought to be produced through various combinations of both nanostructures and pigment (Simon, 1971; Parker, 2002), but as far as we are aware, no data exist to support this hypothesis. Because of their diversity in color and (potentially) color production mechanisms, as well as rich genetic data from breeders, budgerigars may serve as a model system for understanding both physical and genetic bases of color evolution. Here, we use multiple techniques to identify the physical bases of color production in seven morphs of the budgerigar to determine the relative contribution of pigments and structure to plumage color.

MATERIALS AND METHODS

Feather samples and microscopy

We examined contour feathers of seven different budgerigar color morphs, using samples obtained from local pet stores. We washed feathers in distilled water, dried them overnight at 60°C and prepared them for spectroscopy and microscopy.

Spectroscopy

We measured feather reflectance using UV–visible spectrometry. For all measurements, we taped overlaid stacks of three feathers of each color to black velvet. Reflectance was measured from these stacks using an Avantes AvaSpec-2048 spectrometer and an AvaLight-XE pulsed xenon light source, relative to a WS-2 white reflectance standard (Avantes Inc., Boulder, CO, USA). Spectral data were collected at coincident normal (0 deg incident light/0 deg measurement) incidence using a bifurcated micron fiber-optic probe held by a probe holder (RPH-1, Avantes) with a matte black interior that excluded ambient light. We took three measurements from each sample using AvaSoft software v.7.2, with the probe holder completely removed and placed at a different point on the feather surface before each measurement.

Microscopy

To characterize the micro- and nano-structures responsible for the different colors in these feathers, we used light and electron (scanning and transmission; SEM and TEM, respectively) microscopy. We prepared samples following Shawkey et al. (Shawkey et al., 2003). Briefly, we cut feather barbs, washed them in a solution of 0.1% Tween and 0.25 mol l^{-1} NaOH, and fixed them in a 2:3 (v/v) solution of formic acid and ethanol. We then dehydrated the samples in 100% ethanol (twice for 20 min each time) and infiltrated them in 15, 50, 70 and 100% Epon (24 h each time). After curing the blocks at 60°C for 16 h in an oven, we trimmed them with a Leica S6 EM-Trim 2 (Leica Microsystems GmbH, Wetzlar, Germany) and cut 100 nm thin sections using a Leica UC-6 ultramicrotome (Leica Microsystems). We stained these sections with uranyl acetate and lead citrate and viewed them on a Tecnai TEM (FEI, Hillsboro, OR, USA) at an operating voltage of 120 kV. For light microscopy, we cut 1 µm thick sections, transferred them with a loop to glass slides and viewed them on a Leica optical microscope.

Pigment extraction

We extracted the lipid-soluble [likely psittacofulvin (Völker, 1936)] non-melanin pigments from feathers using thermochemical extraction with organic solvent transfer following McGraw et al. (McGraw et al., 2004). Briefly, we placed feathers in tubes with 1 ml acidified pyridine (three drops HCl in 50 ml pyridine) and heated them in a water bath at 95°C for 4 h. The samples were then cooled to room temperature and rinsed with 1 ml distilled water and 5 ml hexane:tert-butymlethylether (1:1, v/v). We included blue feathers of the eastern bluebird (*Sialia sialis*) as a control for the effects of the treatment on color because: (1) they do not contain pigments and (2) their spectral characteristics and their anatomical bases are well known (Shawkey et al., 2003; Shawkey et al., 2005; Shawkey et al., 2009). To quantify spectral changes with treatment, we measured feather reflectance again after the pigment extraction following the same spectral procedure and subtracted post- from pre-treatment reflectance values at each wavelength, creating a 'treatment effect' spectral curve. We determined the absorbance spectrum (from 300 to 700 nm) of the extracted yellow pigment using an absorbance microplate spectrophotometer (Spectramax Plus 384, Molecular Devices Inc., Sunnyvale, CA, USA). We predicted that if pigment removal caused observed changes in feather color in an additive manner (e.g. without changing the refractive index of the substrate material), then this absorbance curve should closely match the treatment effect curve.

Quantitative analysis of barb nanostructure

Both the size and regularity of the keratin and air spaces affect aspects of reflected color (hue and saturation, respectively) (Shawkey et al., 2003; Shawkey et al., 2005). Therefore, we used ImageJ 1.36b (http://rsb.info.nih.gov/ij/index.html) to measure the diameter of keratin rods and air vacuoles present in barbs and to produce profile plots of barb TEM images. The profile plot shows a two-dimensional waveform, representing the spatial density fluctuation in the intensity of pixels (i.e. proxy for spatial variation in refractive index of keratin and air) within a standardized selected area of 3.5×3.5 µm of the TEM image. We calculated the coefficient of variation (CV) of distance between the peaks in the waveform as a measure of nanostructural regularity. We measured spongy layer width at five points, predicting that it would be reduced in barbs lacking a significant structural component to their color.

Fourier analysis

We performed Fourier analysis on TEM images obtained for feather barbs using the Fourier tool for biological nano-optics (Prum and Torres, 2003). For all images, the largest available square portion of keratin and air (>500 pixels) uninterrupted by melanin granules, cell boundaries or keratin cortex was selected. This analysis allows the user to determine whether nanostructures are sufficiently organized at an appropriate scale to produce color by coherent light scattering alone (Prum et al., 1999a; Shawkey et al., 2003).

RESULTS

Spectroscopy

Grey feathers had a typical achromatic spectral curve with no discrete peaks and rapid increase in reflectance at short wavelengths (~300–400 nm; Fig. 1B). The spectral curves of yellow, green, turquoise, light blue and dark blue feathers showed two reflectance peaks, one between 330 and 398 nm and a larger one between 526 and 588 nm (Fig. 1C–G, Table 1). Spectra of these colors fell to a minimum at ~440 nm. Purple feathers had a single peak at 421 nm (Fig. 1H).

Microscopy

All seven colored feather barbs had some amount of spongy medullary keratin in their feather barbs (Fig. 2), but grey and yellow feathers had much less than other colored barbs (Table 2). The size and structure of the spongy layer varied extensively among the colored barbs examined. The diameters of keratin channels and air vacuoles were smallest in purple feathers and largest in white and grey feathers (Table 2). Moreover, the variation in spatial fluctuation was almost twice as high in grey and yellow feathers than in other colors, indicating decreased organization of the spongy layer (Table 2).

All barbs, other than those from yellow and grey feathers, contained a layer of melanosomes basal to the spongy layer and surrounding large central vacuoles (Fig. 2). Melanosomes were only present in the barbules of grey feathers, and were absent altogether in yellow feathers.

Pigment extraction

Yellow pigment was visible in the barbules and cortex of barbs in yellow, green and turquoise feathers (Fig. 2C,D,F). After pigment

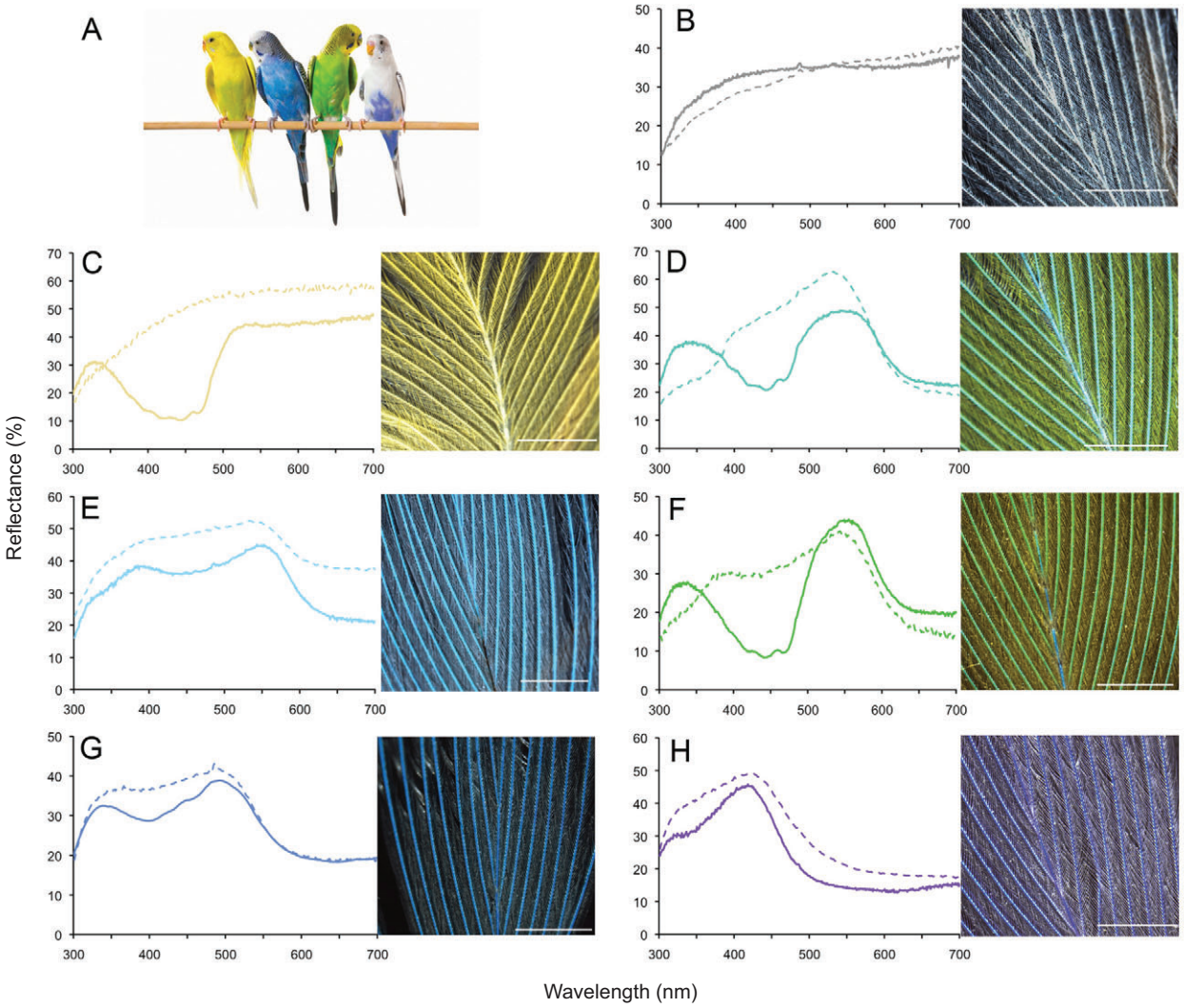

Fig. 1. (A) Various color morphs of budgerigars, *Melopsittacus undulatus*. Photo credit: Corey Hochachka, Design Pics/Corbis. Panels show colored feathers of (B) grey, (C) yellow, (D) turquoise, (E) light blue, (F) green, (G) dark blue and (H) purple morphs and their measured spectra before (solid line) and after (dashed line) pigment extraction. Line colors used correspond to perceived feather hue. Scale bars, 1 mm.

removal, yellow feathers appeared white; in light blue, dark blue, turquoise and green feathers, reflectance from 390 to 480 nm increased, eliminating the characteristic double peak of the spectral curves observed before the extraction (Fig. 1). The extracted pigment showed maximum absorbance between these wavelengths (Fig. 3A), and mirrored the treatment effect curve from yellow, green and turquoise feathers, indicating its central role in production of the double peaks in the colors of these morphs (Fig. 3B). Weaker similarities were also seen in light and dark blue morphs, perhaps because of lower pigment concentrations (Fig. 3B). By contrast, purple and grey budgerigar feathers (Fig. 3B) and the control bluebird feathers (Fig. 3C) showed a small change in reflectance due to the extraction; this change was dissimilar to the pigment absorbance profile, indicating a lack of pigment.

Table 1. Means of spectral variables before and after the pigment extraction of budgerigar feathers

	Before extraction				After extraction			
Color	Brightness (%)	Hue (nm)	Spectral saturation (%)	UV chroma (%)	Brightness (%)	Hue (nm)	Spectral saturation (%)	UV chroma (%)
Grey	29	–	–	22	31	–	–	16
Yellow	22	588	16	24	52	–	–	15
Turquoise	28	554	19	26	38	530	20	18
Light blue	44	543	17	22	31	531	15	21
Green	26	558	23	25	28	560	18	21
Dark blue	31	486	19	24	29	485	16	26
Purple	29	421	23	31	24	418	23	35

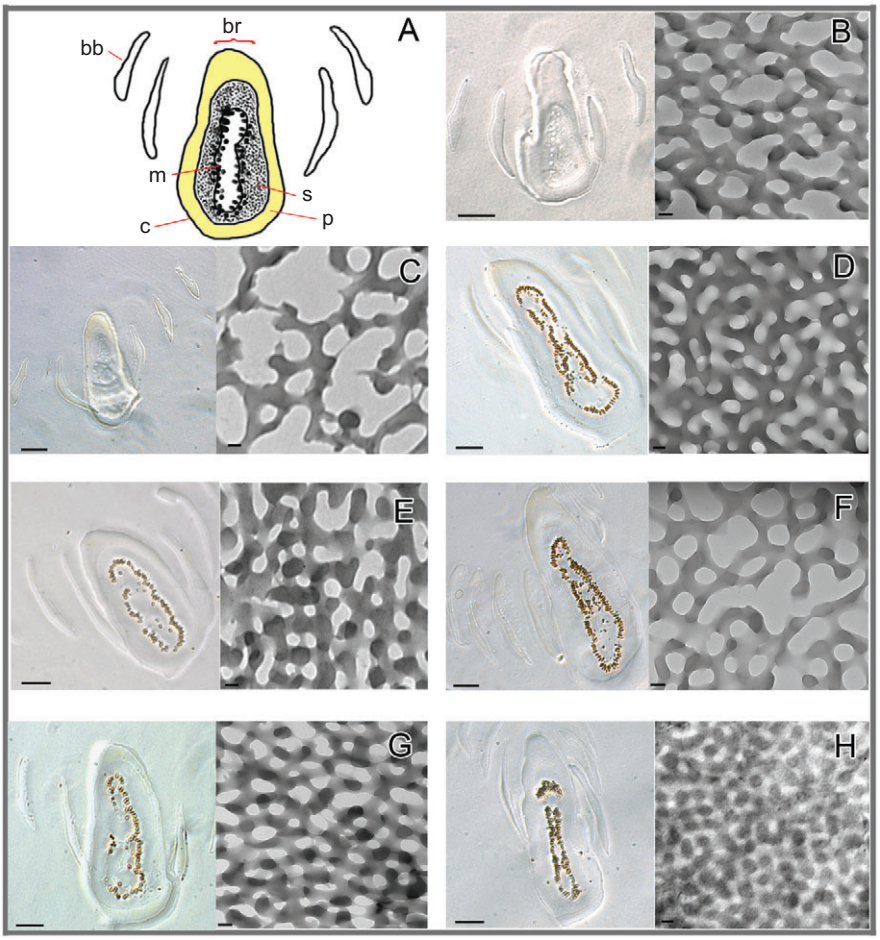

Fig. 2. (A) Schematic of a budgerigar feather barb (br) indicating the location of barbules (bb), barb cortex (c), layer of melanosomes (m), spongy layer (s) and yellow pigment (p). Panels show light microscope and transmission electron microscopy (TEM) images of the spongy layer of (B) grey, (C) yellow, (D) turquoise, (E) light blue, (F) green, (G) dark blue and (H) purple morphs. Scale bars for light microscope images are 10 μm, and 100 nm for TEM micrographs.

Fourier analysis

All feather barbs excluding those from grey and yellow feathers showed discrete rings in the Fourier power spectra (Fig. 4), indicating high levels of nanostructural organization (Prum et al., 1998; Prum et al., 1999b; Prum et al., 1999a). The profile plots of TEM micrographs showed that in yellow and grey morphs, the spatial fluctuations in the refractive index (of keratin and air) were the least regular whereas those of purple barbs were the most regular (Fig. 4, Table 2).

DISCUSSION

We have demonstrated that structures and pigments play more widespread roles in color production in budgerigars than previously thought. Psittacofulvin pigments, thought to be present only in yellow and green feathers (Simon, 1971; Taylor and Warner, 1986; Nemésio, 2001), affect the color of blue and turquoise feathers. Similarly, and in contrast to previous predictions (Simon, 1971), melanosomes are present in light blue feathers and a spongy layer is present in grey feathers. Below we discuss how these components interact to produce colors.

It was proposed nearly 40 years ago that a lack of melanin in structurally colored blue barbs results in a light blue color (Simon, 1971) because the melanin layer served as a black background that darkens the color of the spongy layer. However, Shawkey and Hill (Shawkey and Hill, 2006) showed that removal of melanin leads to an almost complete loss of color. This is because the melanin serves

Table 2. Means ± s.e.m. of nanostructural elements of budgerigar feather barbs

Color	Air vacuole diameter (nm)	Keratin channel diameter (nm)	Spongy layer width (nm)	Inter-peak distance	
				Mean (nm)	CV (%)
Grey	247±11	150±4	3089±428	290	42
Yellow	218±11	138±5	4401±448	332	40
Turquoise	117±3	109±5	5995±599	201	23
Light blue	115±3	104±5	5641±708	229	27
Green	126±3	117±4	6346±386	222	25
Dark blue	121±4	103±4	4980±230	226	21
Purple	87±3	80±3	7204±370	175	16

Predicted hue values calculated from the two-dimensional Fourier power spectra. Means and CV of the distance between peaks were obtained from profile plots of electron micrographs indicating regularity of spatial density fluctuations in refractive index of the barb spongy layer.

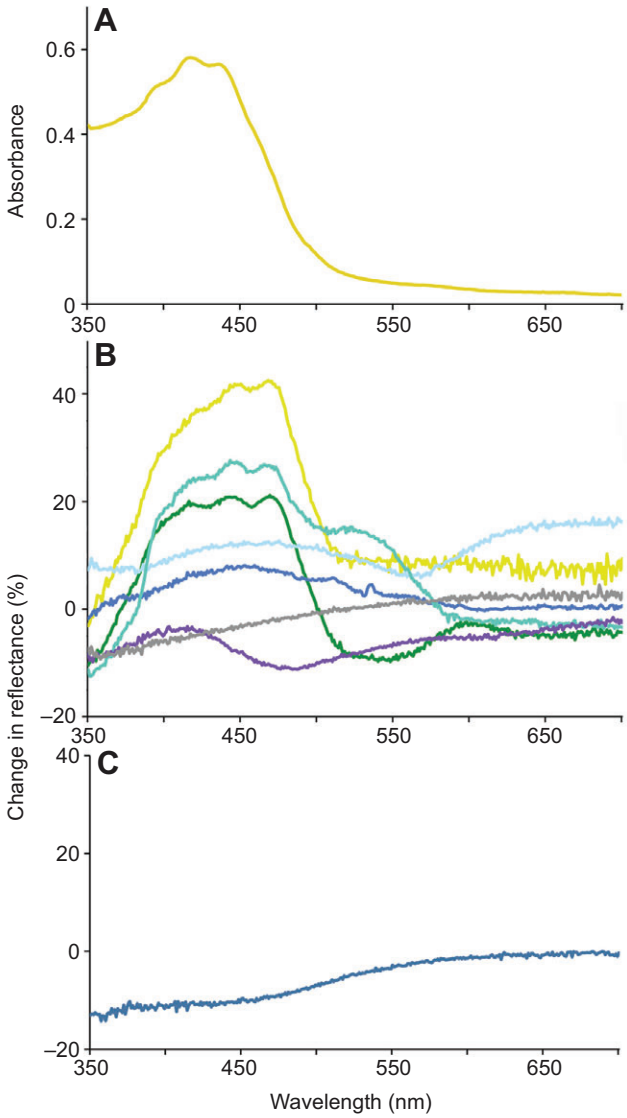

Fig. 3. (A) Absorbance curve of extracted pigment from yellow budgerigar feathers. Note that the wavelength on the *x*-axis starts at 350 nm to eliminate absorbance by pyridine used for extraction (see Materials and methods). Pyridine did not show absorbance above 360 nm (supplementary material Fig. S1). (B) Difference in reflectance before and after the pigment extraction in all seven budgerigar color morphs and (C) eastern bluebird blue feathers.

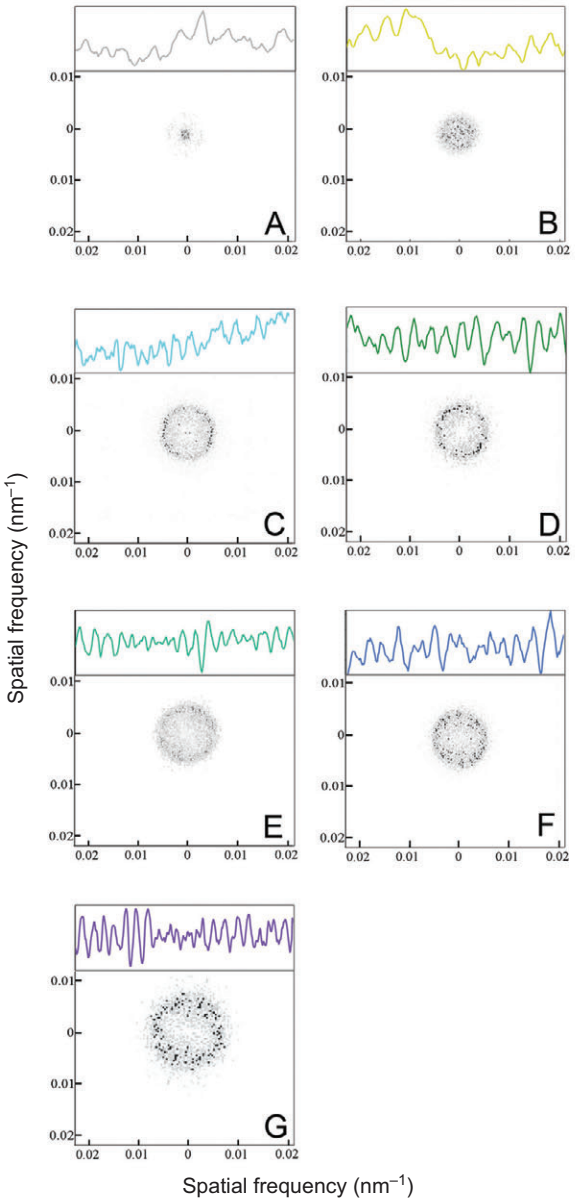

Fig. 4. Two-dimensional Fourier power spectra of TEM of spongy layers in budgerigar feather barbs for (A) grey, (B) yellow, (C) turquoise, (D) light blue, (E) green, (F) dark blue and (G) purple morphs. The waveforms above each power spectrum obtained from TEM profile plots represent spatial fluctuations in dark (keratin) and light (air) areas in the barb tissue (*y*-axis: grey value) within an area of 3500×3500 nm (*x*-axis: distance). Variation in spacing between wave peaks indicates irregularity of spatial fluctuations. Line colors used correspond to perceived feather hue.

to absorb incoherently scattered white light that would otherwise wash out the coherently scattered blue color. Our data show that all feather barbs except for yellow and grey had a basal melanin layer underlying the spongy layer, illustrating that it is critical for proper non-iridescent structural color production, although in some cases melanin may be replaced by carotenoids (Dyck, 1971b). Furthermore, our results suggest that differing shades of blue are the result of slight variations in the regularity of spongy medullary nanostructures. The nanostructural regularity of light blue feathers was lower than that of dark blue barbs (Table 1), likely leading to increased incoherent scattering and thus brighter but less saturated color (Table 2).

Pigments were present in both light and dark blue feathers, but their effect was more pronounced in green and turquoise feathers. Green feathers appear blue to the naked eye after pigment extraction, as would be expected from the traditional view that blue structural color plus yellow pigment leads to a green feather (Parker, 2002). However, spectrometry revealed that the hue of the depigmented feathers remained within the green wavelengths. Moreover, the nanostructural length scale and disorder of green barbs was larger than that of dark blue barbs. The removal of pigments did not change this hue but rather caused enhanced reflection of blue wavelengths and hence loss of green color. Together, these results indicate that the nanostructure of green barbs is 'tuned' to green color (Prum et al., 1999a), but that selective absorption of blue wavelengths by pigments is necessary for it to be perceived as green.

Similarly, the difference between turquoise and light blue colors appears to be attributable at least in part to pigment content. These two color morphs had similar nanostructural length scale and regularity, but the hue of turquoise barbs hypsochromically shifted twice as much after pigment extraction (24 nm *vs* 12 nm), giving both feathers nearly identical hues. This result and the greater increase in reflectance of blue wavelengths following extraction in turquoise feathers indicate that difference in pigment concentration largely explains the difference in color between these two morphs.

These results suggest that lineages utilizing both nanostructures and pigments to produce colors are uniquely capable of color diversification. Non-iridescent green color may not be possible without the use of pigments, either by themselves (e.g. turacoverdin) (Dyck, 1992) or in conjunction with green-tuned spongy layers. Although more extensive sampling is needed, as far as we are aware no spongy layer has been shown to produce a color outside of the UV to blue range without the use of pigments (Prum, 2006). Thus, pigments expand the capabilities of spongy layers, but, potentially, only to a certain extent. The lack of organization and thus coherent light scattering in the yellow morph suggests that the long length scales required for production of longer wavelength colors may be developmentally or physically out of reach for spongy layers [but see Stavenga et al. (Stavenga et al., 2011) for spectral evidence of red spongy layers]. Spongy layers are thought to form through self-assembly processes (Dufresne et al., 2009; Prum et al., 2009) similar to those observed in synthetic polymers that reach stable length scales of up to several microns. However, whether spongy layers formed from beta keratin would be similarly stable is unknown. Interestingly, the disorganized layers of both the grey and yellow feathers also lack melanosomes, suggesting that their presence could help stabilize spongy layers during development. These hypotheses should be tested in future work. This investigation of the anatomical and physical basis of pigmentary and structural interactions thus adds considerably to our understanding of the mechanisms of color production in birds, and sets the stage for future studies of the genetic basis of color variation and evolution.

ACKNOWLEDGEMENTS

We thank R. Maia and C. Eliason for valuable comments on the manuscript. R. Maia provided assistance with electron microscopy.

FUNDING

This work was supported by University of Akron start-up funds and Air Force Office of Scientific Research (AFOSR) grant FA9550-09-1-0159 (both to M.D.S.).

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