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Significance of a basal melanin layer to production of non-iridescent structural plumage color: evidence from an amelanotic Steller's jay (*Cyanocitta stelleri*)

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Summary

Non-iridescent structural plumage color is typically produced by coherent scattering of light within a matrix of keratin and air (a 'spongy layer') in feather barbs. It remains unclear what role, if any, the basal melanin layer underlying this spongy layer plays in the production of coloration. Amelanism in birds with structural color is a 'natural experiment' in which melanin pigmentation is lost, allowing us to identify the effects of the loss of melanin on structural color production. Here we use full-spectrum spectrometry, transmission electron microscopy and Fourier analysis to compare the color and nanostructure of an amelanotic Steller's jay (*Cyanocitta stelleri* Gmelin) feather with a normal blue Steller's jay feather. As a control, we also examined a white domestic chicken (*Gallus gallus* Linnaeus) feather. The pale amelanotic jay feather had a broad reflectance curve with a blue/green peak, while the typical blue feather had a typical distinct bell-shaped blue curve with a UV/violet peak. The white chicken feather had a typical white reflectance curve with no discrete peaks. Electron microscopy revealed that both the amelanotic and blue feather barbs contained well-formed spongy layers that were of the correct size and arrangement to produce their measured peak reflectance values, whereas the chicken feather had no spongy layer. The washed-out color of the amelanotic jay feather was thus most probably caused by the loss of the basal melanin layer, suggesting that melanin functions to absorb incoherently scattered white light from the feather barb thereby increasing the purity of the color produced by the spongy layer.

Key words: melanism, sexual selection, Fourier analysis, feather, Steller's jay, *Cyanocitta stelleri*.

Introduction

Understanding the mechanisms by which color is produced in feathers can lead to insights into their signal content and evolution of color displays. Feather coloration in birds can arise through the deposition of pigments (primarily melanins and carotenoids) or through the precise arrangement of tissues at a nanometer scale (Gill, 1995; Shawkey and Hill, 2005; Hill and McGraw, 2006). The latter form of coloration is referred to as structural coloration, and is typically classified as either iridescent (i.e. varying in hue at different angles of observation) or non-iridescent. Non-iridescent coloration is produced in many cases by coherent scattering of light by highly organized matrices of keratin and air within feather barbs (Dyck, 1971; Dyck, 1976; Prum, 1999; Prum, 2006; Shawkey et al., 2003). This medullary 'spongy layer' lies beneath a keratin cortex and above a layer of melanin granules surrounding large central vacuoles. Whereas this spongy layer has been studied in some detail, the functions of other anatomical features of feather barbs in color production are less well understood. In particular, the role of the melanin layer that underlies the spongy layer in barbs is still unclear.

Two functions have been hypothesized for this melanin layer. The first hypothesis (hereafter referred to as the "absorbance" hypothesis) posits that melanin absorbs incoherently backscattered white light from the vacuoles and thereby lowers reflectance 'noise' to increase the purity of the color reflected by the spongy layer (Prum, 2006). The second hypothesis (hereafter referred to as the 'backdrop' hypothesis) posits that the melanin layer serves as a black backdrop that darkens the color of the spongy layer, an effect proposed to explain color differences between dark and light blue morphs of Budgerigars *Melopsittacus undulatus* (Simon, 1971). Alternatively, melanin may serve no purpose in color production, existing in feathers to enhance rigidity (Burtt, 1979) or resistance to degradation (Goldstein et al., 2004; Shawkey and Hill, 2004) or for some other function.

The amelanotic feathers from individuals of species with structural coloration provide a unique opportunity to test the function of the basal melanin layer in structural color

production. Amelanotic individuals lack melanin because of a disruption in the pathway of melanin synthesis that typically has no effect on other mechanisms in the body (e.g. keratin deposition) (Majerus, 1998). Thus, by comparing amelanotic to normal feathers, we can determine the effect of melanin on the color of these feathers.

We have used full-spectrum spectrometry and transmission electron microscopy (TEM) to compare the color and nanostructure of the feathers of amelanotic white and normal blue Steller's jays (*Cyanocitta stelleri* Gmelin), and the white feathers from a domestic chicken (*Gallus gallus* Linnaeus). We wanted to determine if loss of melanin could explain the shift from blue to white color in the amelanotic feather or if other anatomical differences such as loss of spongy layer were also involved. Thus, we compared the nanostructure of the three feathers using TEM and the Fourier tool for biological nano-optics (Prum and Torres, 2003). This tool allowed us to predict the hue of feathers through analysis of the nanostructural arrangement of the spongy layer. If loss of melanin alone explained the observed color differences, then both the normal and amelanotic feathers should have well-defined spongy layers with predicted hue values in the blue wavelengths. This would suggest that both the amelanotic and blue feathers could produce blue color, but that the lack of melanin granules in the amelanotic feathers prevents the color from being expressed. Alternatively, the barbs of the amelanotic feather could lack a spongy layer and resemble barbs from a normal white feather. The white color of the amelanotic feather could then be explained as a result of a loss of color-producing structures, rather than as a loss of melanin granules *per se*.

Materials and methods

A normally colored Steller's jay was observed molting from species-typical blue into all-white plumage in the back yard of a resident in Boulder County, CO, USA (B. Schmoker, personal communication; see Fig. 1). A white Steller's jay tail feather was retrieved from the ground in this backyard. A blue tail feather was collected from a normally colored male Steller's jay specimen in the Burke Museum (Seattle, WA, USA). For further comparison, a white domestic chicken feather was collected from a pen at the Auburn University Poultry Science Department.

An Ocean Optics S2000 spectrometer (range 250–880 nm; Dunedin, FL, USA) was used to take reflectance measurements from these feathers. Feathers were placed on gloss-free black construction paper, and ~20 mm^2 patches of color approximately three-quarters of the feather's length away from the proximal end of each feather were chosen for analysis. Using a block sheath that excluded ambient light, a bifurcated micron fiber optic probe was held at a 90° angle 5 mm from the feather surface, creating a measurement area of 2 mm in diameter. This measurement area was illuminated by both a UV (deuterium bulb) and a visible (tungsten–halogen bulb) light source. All data were expressed relative to a white standard (WS-1, Ocean Optics). OOIbase software was used to record and average 20 spectra sequentially, and these spectra were recorded and averaged from five arbitrarily chosen points within the selected locations on each feather.

Fig. 1. Photograph of the amelanotic Steller's jay in Boulder County, CO, USA from which the feathers in this study were taken. Photograph by Bill Schmoker.

From these reflectance curves several different color variables were calculated. These indices were restricted to wavelengths between 320 and 700 nm, as evidence suggests that passerine birds are sensitive to ultraviolet (UV) wavelengths (Cuthill et al., 2000), and that 700 nm is the upper limit of the vertebrate visual system (Jacobs, 1981). The wavelength of maximum reflectance was used as an index of hue, the principal color reflected by the feathers (Keyser and Hill, 1999). Brightness, the sum of reflectance from 320–700 nm, is a measure of the total amount of light reflected by the feathers (Andersson, 1999). Ultraviolet (UV) and blue chromas are the summed reflectances of light in the ranges of 320–400, and 435–500 nm, respectively, divided by brightness, and are indices of color purity (Andersson et al., 1998).

Feather barbs from the amelanotic and the blue Steller's Jay and the white chicken were prepared for transmission electron microscopy (TEM) following the methods of Shawkey et al. (Shawkey et al., 2003) and viewed on a Phillips EM301 TEM (Veeco FEI Inc, Hillsboro, OR, USA). Micrographs of feather barbs and a waffle-pattern diffraction grating (Ted Pella, Redding, CA, USA) accurate to 1 nm ±5% were taken at the same magnifications for calibration of the images.

TEM micrograph negatives were scanned at 400 d.p.i. using an Epson Perfection^{TM} 1240U flatbed scanner. These micrographs were then analyzed using Prum and Torres' Fourier analysis program for biological nano-optics (Prum and Torres, 2003). This MATLAB-based program uses Fourier analysis to determine whether the spongy layer of feather barbs is sufficiently organized, and at an appropriate scale, to produce color by coherent light scattering alone (Prum et al., 1999; Prum et al., 1998). Subsequent radial analyses incorporating the estimated refractive indices of keratin

(RI=1.56) and air (RI=1.00) allow the user to obtain a predicted hue. For all analyses, the largest available square portion of spongy layer (>500 pixels) uninterrupted by melanin granules, cell boundaries or keratin cortex was selected. Because the barbs of white chicken feather lacked spongy layers, the central vacuoles and the keratin surrounding them were selected for analysis.

Because other microanatomical features of barb morphology other than the spongy layer may affect color production (Shawkey et al., 2005) the program NIH Image version 1.62 (available for download at http://rsb.info.nih.gov/nih-image) was used to measure additional structural components of the two colored barbs. The thickness of the keratin cortex and spongy layer was measured at six different, evenly spaced points around the barb. Barbs from the white chicken and jay feathers contained no melanin, so we could not measure density or size of melanin granules for these feathers.

Results

To the human eye, under diffuse mid-day lighting, the chicken feather was white with no overtones, the amelanotic jay feather was white with a faint blue overtone, and the normal jay feather was dark blue with black stripes. The reflectance spectrum of the chicken feather was similar to that of the white feathers of other species of birds (Mennill et al., 2003; Shawkey and Hill, 2005) with uniformly high reflectance across all wavelengths tailing off at short wavelengths and with no discrete peaks. The reflectance spectrum of the amelanotic feather was similar to that of the white feather (Fig. 2). Unlike the white feather of a chicken or chickadee (Mennill et al., 2003), however, the amelanotic feather had a single shallow peak in the blue/green range followed by gradually decreasing reflectance. The reflectance spectrum of the blue portion of the typical Steller's jay feather was similar to that of other species with non-iridescent blue coloration (Dyck, 1971; Prum et al., 1999; Shawkey et al., 2003), with a bell shape and a peak in the UV/violet range (Fig. 2).

The hue of the amelanotic jay feather was shifted about 100 nm longer compared to that of the blue Steller's Jay feather, in the low end of the green wavelengths (Table 1). Brightness of the blue feather was dramatically lower than that of the amelanotic and white feathers, while UV chroma and blue chroma of the blue feather were somewhat higher (see Table 1).

The microanatomy of the blue feather barbs was similar to that of barbs of other species producing non-iridescent blue

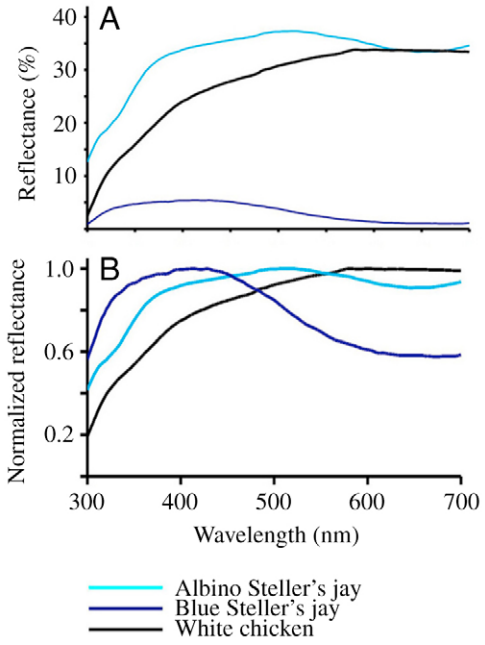

Fig. 2. Raw (A) and normalized (B) reflectance spectra of an amelanotic Steller's jay feather, a normal blue Steller's jay feather (solid line) and a white chicken feather. Reflectance values in B were all divided by the peak reflectance value of each curve to obtain a maximum reflectance of 1. The spectra are presented both ways to facilitate comparison of both overall reflectance (A) and the shapes of the curves for the different feathers (B).

structural color (Fig. 3A) (e.g. Dyck, 1971; Shawkey et al., 2003). The medullary spongy layer sat beneath a fairly thick keratin cortex and above a single layer of melanin granules surrounding hollow central vacuoles. This spongy layer was composed of a matrix of irregularly shaped keratin and air 'bars', resembling the structure observed in the blue feathers of peach-faced lovebirds *Agapornis roseicollis* (Dyck, 1971), Eastern bluebirds *Sialia sialis* (Shawkey et al., 2003) and others (see Prum, 2006). The amelanotic feather differed from this blue feather in two ways (Fig. 3A,B). First, the basal layer of melanin granules was absent. Second, the keratin cortex of the amelanotic feather was considerably thicker than that of the blue feather (Fig. 3A,B; Table 1). In the white chicken feather, the spongy layer and melanin layer were completely absent, but the cortex was about as thick as that of the blue feather (Fig. 3C; Table 1).

Fourier analysis revealed that the spongy layers of the blue and amelanotic feather barbs were sufficiently organized and

Table 1. *Color and morphological measurements of an amelanotic Steller's jay feather, a normal blue Steller's jay feather and a white chicken feather*

Feather	Hue (nm)	Predicted hue (nm)	Brightness (%)	Chroma (%)		Thickness (nm)	
				UV	Blue	Spongy layer	Cortex
Blue	413	441	8.08	27.20	33.28	4474.41	8383.77
Amelanotic	514	493	37.93	20.37	30.37	6313.93	12052.33
White	n/a	n/a	32.94	15.92	28.81	n/a	7386.47

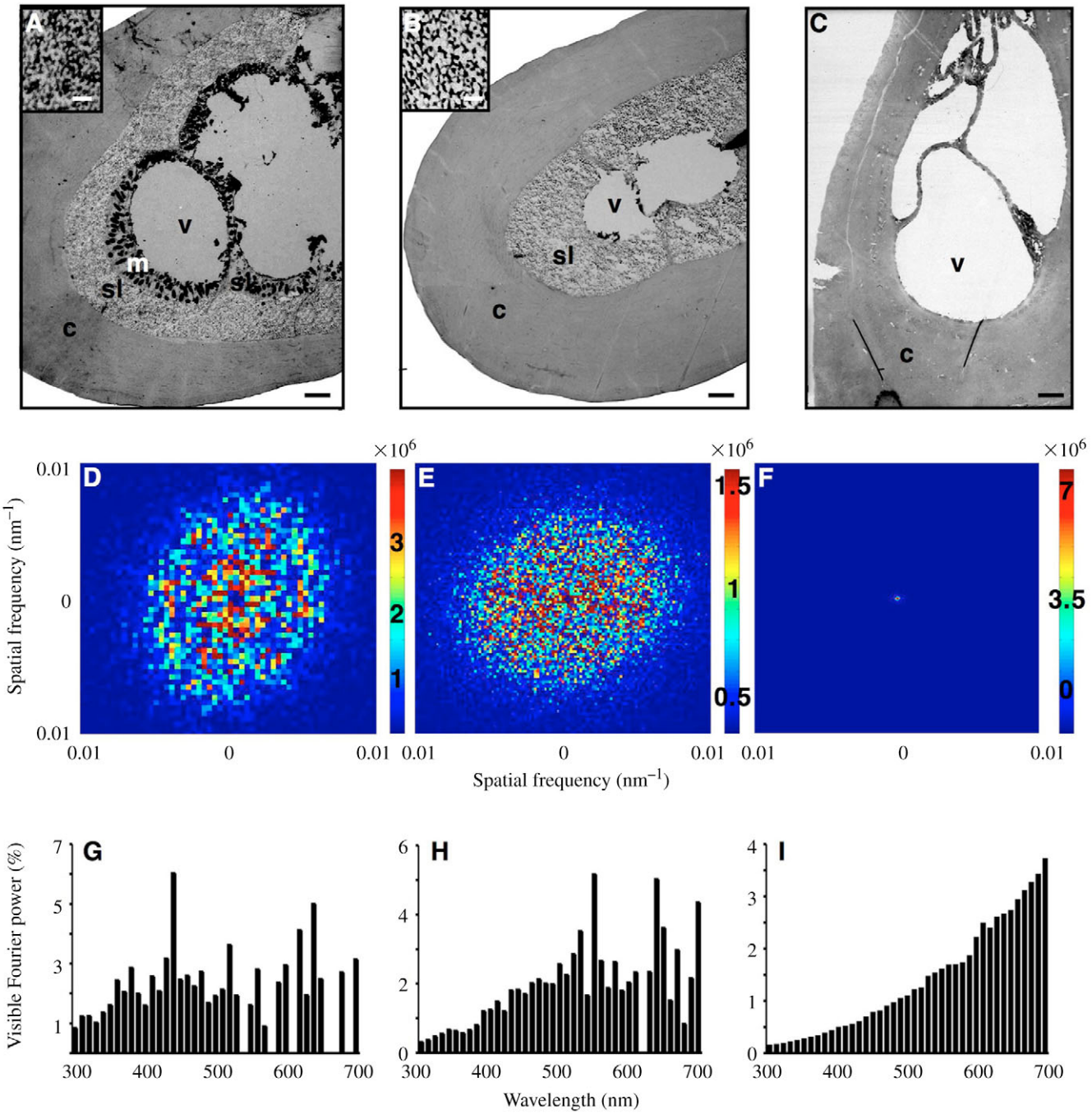

Fig. 3. Feather microstructure, Fourier power spectra and predicted reflectance spectra of a blue Steller's Jay feather (A,D,G), an amelanotic Steller's jay feather (B,E,H) and a white chicken feather (C,F,I). (A–C) TEM micrographs of barbs (scale bars, 1 μm) with insets in A and b showing close-ups of spongy layer (scale bars, 500 nm). (D–F) Two-dimensional Fourier power spectrum of TEM of colour-producing structures in these feathers. The length and direction, respectively, of a vector from the origin to each value in the power spectrum indicates its spatial frequency and direction. The colour indicates the magnitude of each value in the power spectrum (scale on right). (G–I) Predicted reflectance spectra based on these Fourier analyses and the known refractive indices of keratin (1.54) and air (1.0) (for details, see Prum and Torres, 2003). c, cortex; sl, spongy layer; m, melanin granules; v, vacuoles.

at the correct scale to produce color by coherent light scattering alone. The discrete rings in the Fourier power spectrums (Fig. 3D,E) indicate high levels of nanostructural organization (Prum et al., 1998; Prum et al., 1999). Fourier analysis of the spongy layer of these two feathers predicted hue values close to measured values (Fig. 3G,H; Table 1). The predicted hue for the amelanotic feather was 21 nm shorter than the measured hue and the predicted hue for the blue feather was 28 nm longer than the measured hue (Table 1). This degree of error is comparable to that seen in other studies using this tool (Prum

et al., 2003). By contrast, the Fourier power spectrum of the chicken feather showed no discrete shape and very low power, indicating a lack of nanostructural organization (Fig. 3F). This lack of organization results in a lack of discrete peaks in the radial analysis (Fig. 3I).

Discussion

The role of melanin in the production of structural coloration has been successfully modeled in non-avian vertebrate taxa (Grether et al., 2004), but as far as we are aware, this is the first study to examine the effects of loss of melanin on the production of blue structural color in feather barbs. Whereas the role of melanin in non-iridescent avian structural color production has been the subject of speculation (Simon, 1971; Prum, 1999; Prum, 2006), no tests of the function for this melanin layer have been conducted. Our visual assessment, spectrometric measurements and nanostructural analyses of the white feathers of an amelanotic Steller's jay all support the hypothesis that melanin primarily functions to absorb incoherently scattered white light from feather barbs. First, the amelanotic feather was white with faint blue overtones suggesting that loss of melanin has a clear and dramatic effect on structural color production, refuting the predictions of the null hypotheses. Our spectrometric analyses provide more quantitative data on this effect. If loss of melanin simply lightened the blue color of the feather, as predicted by the backdrop hypothesis, we would expect the reflectance curve of the amelanotic feather to have a peak but have higher reflectance values than the blue feather. Instead, the reflectance spectra of the blue feather has a peak, whereas that of the amelanotic curve is fairly saturated across all wavelengths and has only the suggestion of a peak in the blue/green wavelengths. The higher UV chroma value of the blue feather further suggests that it reflects a more pure, saturated color. However, these observations leave open the possibility that additional changes in the microstructure of the amelanotic feather barbs, such as loss of spongy layer, could explain its washed-out appearance.

Our microstructural analyses, however, indicate that the amelanotic feather has a well-defined spongy layer that is organized at the proper scale to produce a blue/green color. The fact that this amelanotic feather lacks blue/green coloration and appears white to the human eye suggests that the loss of melanin from the barb allows non-specific white reflectance to swamp out the blue color. The absorption of light by the underlying melanin granules in the blue feather thus appears to be essential for expression of blue coloration.

The thicker keratin cortex of the amelanotic barb may also contribute to the observed differences in reflectance. Previous research suggests that the cortex primarily absorbs light in non-iridescent structural plumage color (Finger, 1995). The thicker cortex of the amelanotic feather could therefore reduce the amount of white light reflected. However, the estimated extinction coefficient (a measure of light absorption properties) of melanin is about 20 times higher than that of keratin (Brink and van der Berg, 2004), and thus any absorption by the thicker keratin cortex would be negligible compared to that of melanin.

The low brightness of blue feathers may also largely be caused by absorption of light by the melanin layer within blue barbs, as predicted by both the backdrop and absorbance hypotheses. Although it may seem obvious that barbs containing melanin will reflect less light than barbs without melanin, the absorption of light by melanin has been rarely considered in mechanistic studies of structural plumage color (Greenewalt et al., 1960; Land, 1972). Interspecific differences in brightness and other color variables of both iridescent and non-iridescent structural ornaments may be affected by the presence and concentration of melanin within barbs. Indeed, in a recent study (Brink and van der Berg, 2004), it was shown that the coppery iridescence of feathers of the Hadeda ibis *Bostrychia hagedash* could not be properly predicted by thin-film models without taking the absorbance of melanin into account. Here, we present data suggesting that this absorbance may also play an important role in the production of non-iridescent structural plumage color.

Our results also suggest that melanin density affects the brightness of individual birds; however, in another study we found that density of melanin granules was not correlated with brightness among individual eastern bluebirds (Shawkey et al., 2005). Further studies are needed to determine whether variation in melanin density among individuals affects brightness of structurally colored feathers.

Our small sample size (N=1 for each group) clearly warrants caution in the interpretation of our results. However, because we were observing the effects of complete removal of melanin, many of our conclusions are inescapable. Melanin absorbs incoherently scattered light, increasing color purity, and also darkens feathers. Similar comparisons of normal and amelanotic individuals of other vertebrate taxa (e.g. fish, frogs) with three-layer dermal chromatophore units (Grether et al., 2004) would provide significant insight into the role that melanin plays in this type of structural color. More theoretical and empirical work on the mechanisms that create structural color display is needed. In birds, the development of explicit physical models incorporating all aspects of barb structure will greatly improve our understanding of the mechanics of structural color production. Understanding the proximate role of melanin in structural color production will help us understand how the basic components of almost every feather (keratin, air and melanin) have been modified over evolutionary time to create the amazing diversity of structural color found in birds.

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