JOURNAL OF THE ROYAL SOCIETY Interface

J. R. Soc. Interface (2006) **3**, 527–532
doi:10.1098/rsif.2006.0111
Published online 9 February 2006

Mechanisms of evolutionary change in structural plumage coloration among bluebirds (*Sialia* spp.)

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Combinations of microstructural and pigmentary components of barbs create the colour displays of feathers. It follows that evolutionary changes in colour displays must reflect changes in the underlying production mechanisms, but rarely have the mechanisms of feather colour evolution been studied. Among bluebirds in the genus *Sialia*, male rump colour varies among species from dark blue to light blue while breast colour varies from blue to rusty. We use spectrometry, transmission electron microscopy and Fourier analysis to identify the morphology responsible for these divergent colour displays. The morphology of blue rump barbs is similar among the three species, with an outer keratin cortex layer surrounding a medullary 'spongy layer' and a basal row of melanin granules. A spongy layer is also present in blue breast barbs of mountain bluebirds *Sialia currucoides* and in rusty breast barbs of western *Sialia mexicana* and eastern bluebirds *Sialia sialis*. In blue barbs melanin is basal to the spongy layer, but is not present in the outer cortex or spongy layer, while in rusty barbs, melanin is present only in the cortex. The placement of melanin in the cortex masks expression of structural blue, creating a rusty display. Such shifts in microstructures and pigments may be widespread mechanisms for the evolutionary changes in the colours of feathers and other reflective structures across colourful organisms.

Keywords: structural colour; pigmentary colour; Fourier analysis; coherent light scattering

1. INTRODUCTION

Coloration in animals is thought to arise through two distinct mechanisms. Pigment-based colours are created by the deposition of carotenoids, melanins or other pigments, while structural colours are created through the precise arrangement of tissues at a nanostructural scale (Fox & Vevers 1960; Gill 1995; Hill & McGraw 2006). Structural coloration of the integument, including that of feathers, butterfly scales and invertebrate shells, can produce white, iridescent (Newton 1704) or non-iridescent colour by a variety of mechanisms (Parker 1998; Vukusic & Sambles 2003; Prum 2006).

Microstructures and pigments have traditionally been considered distinct mechanisms of colour production in birds (Hill & McGraw 2006), but recent work shows that microstructural and pigmentary components of feather barbs combine to create colour displays in many bird species (Shawkey & Hill 2005, in press; Doucet *et al.* 2006). Moreover, changes in pigment deposition can cause changes in structural colour at both the individual (Shawkey & Hill in press) and population levels (Doucet *et al.* 2004). However, these changes have not yet been shown to be the basis for changes in species-typical coloration. By examining the morphology associated with distinct plumage colours, we can test the idea that changes in distribution of pigments within feathers can cause major changes in colour display, i.e. from structural to pigmentary coloration, across different species.

Bluebirds in the genus *Sialia* have different expressions of feather coloration. Eastern bluebirds (*Sialia sialis*) have deep blue back and rump feathers with light rusty breast feathers, while mountain bluebirds (*Sialia currucoides*) have pale blue back and rump feathers with faintly blue breast feathers (Sibley 2003). Western bluebirds (*Sialia mexicana*) are intermediate in coloration with blue back and rump feathers that are lighter than eastern bluebirds but darker than mountain bluebirds and rusty breast feathers that are darker than eastern bluebirds. These three species are similar

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Received 17 December 2005
Accepted 10 January 2006

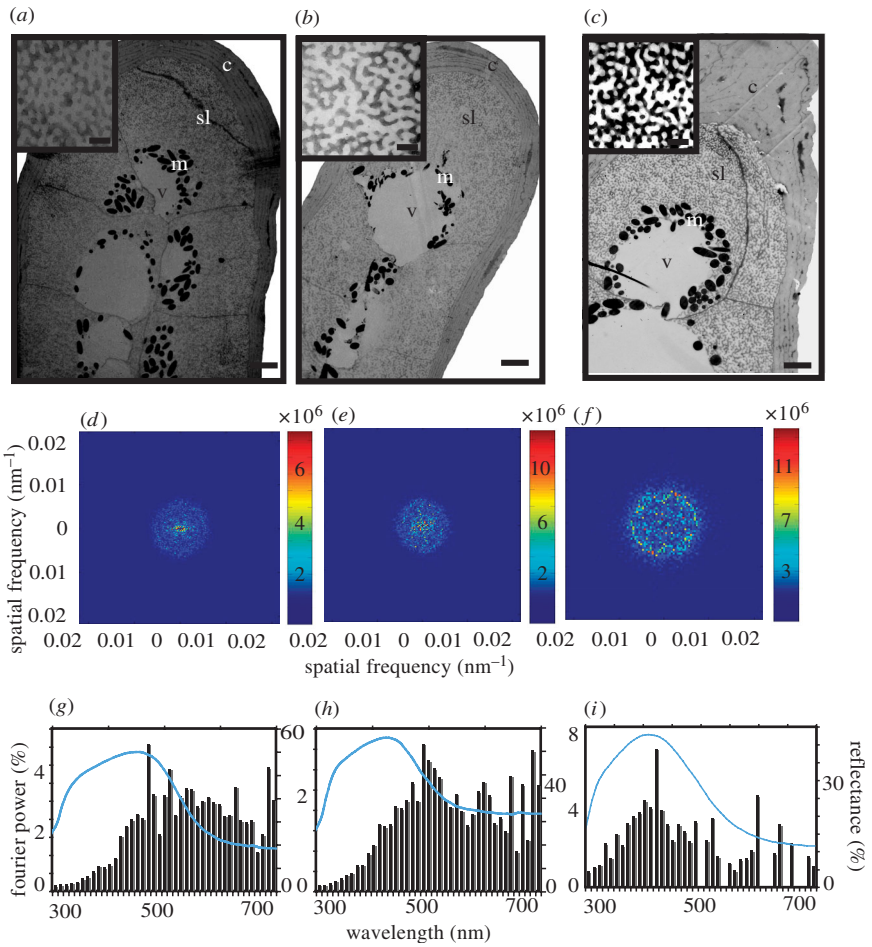

Figure 1. Feather microstructure, Fourier power spectra and measured (curve) and predicted (bars) reflectance spectra of blue rump feathers of mountain bluebirds *Sialia currucoides* (*a*, *d*, *g*), western bluebirds *Sialia mexicana* (*b*, *e*, *h*) and eastern bluebirds *Sialia sialis* (*c*, *f*, *i*). Shown are TEM micrographs of barbs (×1900, *a–c*; scale bar, 1 µm) with insets showing close-ups of spongy layer (×9100, *a–c*; scale bar, 500 nm), Fourier power spectra (*d–f*) and predicted reflectance spectra based on Fourier analysis (*g–i*). C, cortex; SL, spongy layer; M, melanin granules; V, vacuoles.

in overall morphology and ecology and likely form a monophyletic group of fairly recent origin in the Mid-Pliocene (Klicka *et al.* 2005). Genus *Sialia* is thus an ideal taxon on which to investigate the mechanisms that can cause species-typical colour displays. Here we use spectrometry, transmission electron microscopy (TEM) and Fourier analysis to identify morphological differences associated with differences in plumage colour between these species. We hypothesized that changes in blue hue between different species were caused by changes in dimensions of the colour-producing 'spongy layer' of feather barbs, while shifts between blue and rusty colour were caused by differences in melanin pigment deposition.

2. MATERIAL AND METHODS

2.1. Feather sampling

Rump and breast feathers of males of all three species were plucked from live birds as part of separate long-term projects in the USA. We removed 6–8 contour feathers from the rump and breast of each bird. Feathers of eastern bluebirds *S. sialis* were collected from Lee County, Alabama (32°35′ N, 82°28′ W) in March 2003. Feathers of mountain bluebirds *S. currucoides* were collected from a population in Sheridan County, Wyoming (44°46′ N, 107°32′ W) in June 2004 and feathers of western bluebirds *S. mexicana* were collected from the Willamette Valley region of northwestern Oregon (45°18′ N, 122°58′ W) in June 2002. Rump and breast feathers from three individual birds of each species were randomly selected from each feather set for analysis.

2.2. Spectral analysis

For colour analysis, we used the methods of Shawkey *et al.* (2003). Briefly, we taped feathers in stacks of five directly on top of one another to black construction paper, and recorded spectral data from five haphazardly chosen points on the blue portion of these feather stacks using an Ocean Optics S2000 spectrometer (range 250–880 nm, Dunedin, FL, USA) with a bifurcated micron fibre optic probe held at a 90° angle 5 mm from the feather surface using a probe holder (RPH-1, Ocean Optics, Dunedin, Florida). The reading area of 2 mm diameter of light was illuminated by both a UV (D-2000 deuterium bulb, Ocean Optics, Dunedin, Florida) and a visible (tungsten-halogen bulb) light source. All data were generated relative to

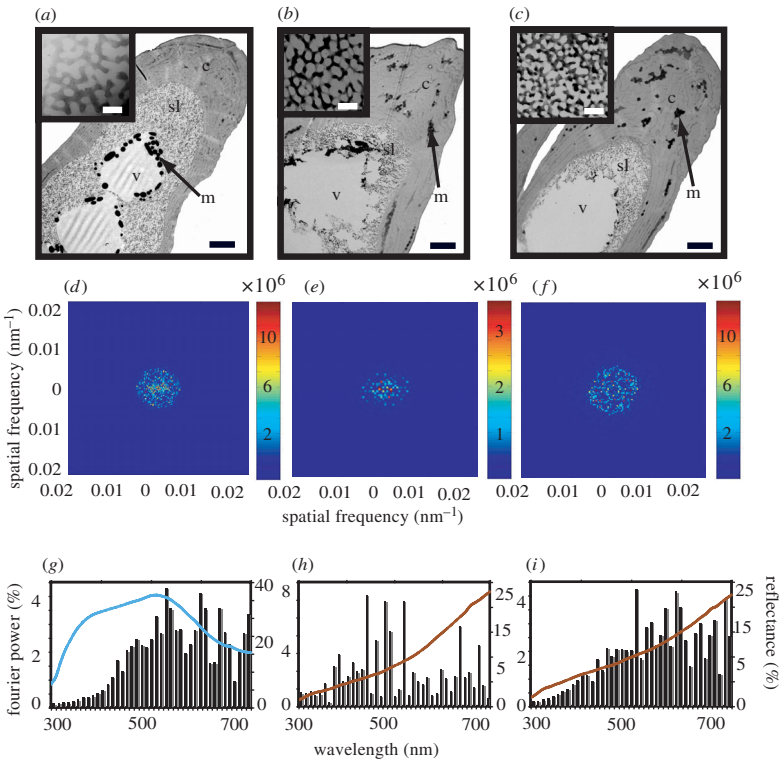

Figure 2. Feather microstructure, Fourier power spectra and measured (curve) and predicted (bars) reflectance spectra of blue or rusty breast feathers of mountain bluebirds *Sialia currucoides* (*a*, *d*, *g*), western bluebirds *Sialia mexicana* (*b*, *e*, *h*) and eastern bluebirds *Sialia sialis* (*c*, *f*, *i*). Shown are TEM micrographs of barbs (×1900, *a–c*; scale bar, 1 µm) with insets showing close-ups of spongy layer (×9100, *a–c*; scale bar, 500 nm), Fourier power spectra (*d–f*) and predicted reflectance spectra based on Fourier analysis (*g–i*). C, cortex; SL, spongy layer; M, melanin granules; V, vacuoles.

a white standard (WS-1, Ocean Optics, Dunedin, Florida).

We calculated the standard colour variables hue (λ_{max}), brightness ($\sum R_{320–700\ nm}$) and UV chroma ($\sum R_{320–400\ nm}/R_{320–700\ nm}$) from these curves (Andersson *et al.* 1998).

2.3. Electron microscopy

Feather barbs were prepared for TEM following the methods of Shawkey *et al.* (2003). Micrographs were taken of each barb ($n=3$ barbs for each bird) at two magnifications: one of the entire barb at 2500× and one of both the cortex and spongy medullary layer at 25 000× magnification.

TEM micrograph negatives were scanned at 400 dpi using an Epson Perfectiong 1240U flatbed scanner. These micrographs were then analysed using Prum & Torres' (2003) Fourier analysis program for biological nano-optics. This MATLAB-based program uses Fourier analysis to determine whether the spongy layer of feather barbs is sufficiently organized at an appropriate scale to produce colour by coherent light scattering alone (Prum *et al.* 1998, 1999). 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 (above 500 pixels) uninterrupted by melanin granules, cell boundaries or keratin cortex was selected.

We used the program NIH Image v. 1.62 (available for download at http://rsb.info.nih.gov/nih-image) to measure the thickness of the keratin cortex and spongy layer at six different evenly spaced points around the barb. We also noted the presence or absence of melanin beneath the spongy layer or in the cortex.

3. RESULTS

3.1. Spectral analysis

Reflectance spectra of rump feathers of all three species exhibited the bell-shaped curves typical of blue structural plumage colour (figure 1*g–i*; Shawkey *et al.* 2003). The reflectance spectra of eastern bluebirds peaked at the shortest wavelengths, while mountain and western bluebirds peaked at longer wavelengths (table 1). Reflectance spectra of breast feathers of eastern and western bluebirds gradually increased with wavelength and showed no discrete reflectance peaks, as it is typical of rusty and brown plumage colours (figure 2*h*,*i*; see Siefferman & Hill 2003). By contrast, spectra of breast feathers of mountain bluebirds were similar to those of rump feathers, with bell-shaped curves and discrete reflectance peaks in the blue/green wavelengths (figure 2*g*; table 1).

3.2. Structural analyses

As expected, the microanatomy of blue feather barbs from the rumps of all three species, as well as blue feather barbs of the breasts of mountain bluebirds was similar to that of barbs of other species producing non-iridescent blue structural colour (figures 1*a–c* and 2*a*; e.g. Dyck 1971; Shawkey *et al.* 2003). The

Table 1. Colour and nanostructural measurements of rump and breast feathers of three species of bluebirds in the genus *Sialia*. (n/a indicates that reflectance curves had no discrete reflectance peaks.)

	hue (nm ± 1 s.e.)	Fourier-predicted hue (nm ± 1 s.e.)	brightness (% ± 1 s.e.)	UV chroma (% ± 1 s.e.)	cortex thickness (nm ± 1 s.e.)	spongy layer thickness (nm ± 1 s.e.)	melanin beneath spongy layer?	melanin in cortex?
rump colour								
Sialia currucoides mountain bluebird	442.40 ± 7.51	460.23 ± 5.50	32.84 ± 1.29	30.27 ± 0.41	3073.80 ± 326.79	5739.66 ± 620.81	y	n
Sialia mexicana western bluebird	412. 20 ± 6.35	461.89 ± 6.23	26.59 ± 1.98	35.48 ± 0.75	2421.06 ± 306.12	6069.88 ± 996.33	y	n
Sialia sialis eastern bluebird	401.10 ± 3.60	419.03 ± 5.52	20.12 ± 0.92	45.00 ± 1.30	2795.32 ± 312.21	4903.32 ± 410.00	y	n
breast colour								
Sialia currucoides mountain bluebird	500.72 ± 3.32	525.23 ± 4.43	19.81 ± 0.89	23.04 ± 1.59	4301.65 ± 907.84	5309.73 ± 916.19	y	n
Sialia mexicana western bluebird	n/a	508.63 ± 3.98	9.58 ± 0.47	8.89 ± 0.55	4971.48 ± 1683.31	5430.60 ± 2714.26	n	y
Sialia sialis eastern bluebird	n/a	435.32 ± 4.35	13.32 ± 3.45	10.32 ± 1.11	4550. 63 ± 539.21	4970.00 ± 230.00	n	y

medullary spongy layer sat beneath a 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' (see Prum 2006 for further examples of this morphology).

Interestingly, the feather barbs of rusty breasts of western and eastern bluebirds also contained spongy layers. These barbs, however, differed from blue barbs in three ways. First, the basal layer of melanin granules was absent (figure 2*b*,*c*; table 1). Second, melanin granules were present in the outer keratin cortex (figure 2*b*,*c*; table 1). Finally, although the spongy layers of the three species were similar in thickness (table 1), they appeared more degraded in the eastern and western bluebird than in the mountain bluebird (figure 2*a*–*c*). The cortices of all feather barbs were too thick to function in thin-film reflection (Vašíček 1960; table 1). What role, if any, this cortex plays in colour production is not known at this point, although some evidence indicates that it may absorb light (Finger 1995; Shawkey *et al.* 2005).

Fourier analysis revealed that the spongy layers of the blue and rusty feather barbs were sufficiently organized and at the correct scale to produce colour by coherent light scattering alone. The discrete rings in the Fourier power spectra (figures 1*d*–*f* and 2*d*–*f*) indicate high levels of nanostructural organization (Prum *et al.* 1998, 1999). Fourier analysis of the spongy layer of blue feathers predicted hue values close to measured values (figures 1*g*–*i* and 2*g*; table 1). Predicted hue values of rusty feathers were also in the blue wavelengths; however, the measured spectra lacked discrete reflectance peaks (figure 2*h*,*i*; table 1).

4. DISCUSSION

The differences in feather coloration among *Sialia* bluebirds are likely of relatively recent origin, because taxa in this genus appear to have diverged from a proto-*Sialia* ancestor in the Mid-Pliocene (Klicka *et al.* 2005). We sought to understand the mechanistic bases for the evolution of species-typical colour displayed by males in the three species in this genus.

Fourier analysis indicated that differences in hue of blue feathers were caused by differences in the dimensions of the medullary spongy layer; for the blue feathers from males of all three species, the hues predicted by Fourier analysis were close to measured values. By contrast, whether feathers were blue or rusty appears to result from differences in the distribution of melanin granules within feather barbs. All blue barbs contained a basal layer of melanin granules, which appears to be necessary for production of non-iridescent structural colour (Parker 2000; Grether *et al.* 2004; Hill & McGraw 2006; Shawkey & Hill in press), and had no melanin in their outer keratin cortex. Rusty barbs, however, lacked this basal melanin layer and had melanin granules scattered within the cortex. In addition, their spongy layers appeared to be degraded in comparison to blue barbs.

These anatomical differences likely affect colour production in distinct ways. In feather barbs that reflect the typical blue colour, the basal melanin layer absorbs incoherently scattered light and thereby allows full expression of the colour created by coherent scattering in the spongy layer (Prum 2006; Shawkey & Hill in press). When this layer is absent, incoherent scattering of light below the spongy layer creates a highly diffuse reflectance spectrum and produces white coloration (Shawkey & Hill in press). By contrast, melanin granules in the outer cortex absorb significant amounts of light before it reaches the spongy layer. The colour observed in rusty bluebird feathers is thus likely caused by the suppression of blue colour production by incoherent scattering and the absorption of light by the outer melanin granules. This pattern is comparable to that seen in blue and black populations of white-winged fairy wrens (*Malurus leucopterus*; Doucet *et al.* 2004). White-winged fairy wrens that are black retain a basal melanin layer but have dense depositions of melanin both within the spongy layer and in the outer cortex.

These results suggest that the rusty breast plumage colour and the associated deposition of melanin in the cortex of western bluebirds is ancestral (Klicka *et al.* 2005), and hence suggest that the deposition of melanin below the spongy layer is derived in this genus. The rusty breasts of some members of the closely related genera *Myadestes* and *Neocossyphus* also imply that this coloration may have been present in the proto-*Sialia* ancestor. However, it is curious that the spongy layer, which appears to serve no function in rusty colour production, is present in the basal taxon western bluebirds. Whether this layer has been lost and gained multiple times in the larger *Turdinae* lineage is an interesting question that could be addressed by more intensive sampling.

Both rump and breast feathers of bluebirds contain spongy layer, but differ in their melanin content. Point mutations in the *melanocortin-1 receptor* gene have been shown to be associated with patterns of melanin deposition in several bird groups (reviewed in Mundy 2005). The localization of variation in melanin deposition in bluebirds (i.e. on the breast but not the rest of the body) suggests that patterns of gene expression, rather than changes in the gene itself, may be responsible. A study of gene expression in developing breast and rump feathers of these three species would add significant insight into the genetic basis of plumage colour expression.

We have shown here that structural colour can evolve both through changes in structures themselves and through a loss and rearrangement of melanin granules. Such simple shifts may be widespread mechanisms for the evolution of colour displays in feathers and other reflective structures across colourful organisms.

We thank M. Toivio-Kinnucan for preparing and sectioning feather barbs for TEM, R. Montgomerie for allowing us to use his colour processing programs and Steve Beissinger's lab group and three anonymous reviewers for helpful comments on the manuscript. This work was funded in part by a Chapman Memorial grant from the American Museum of Natural History to M.D.S., NSF grant IBN-0316541 to L.S.J. and NSF grants DEB-9977039 and IBN-0235778 to G.E.H. M.D.S. was supported during the writing of this manuscript by NSF grant IOB-0517549.

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