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The anatomical basis of sexual dichromatism in non-iridescent ultraviolet-blue structural coloration of feathers

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Despite extensive research on the evolution of avian dichromatism, the anatomical bases for differences between the sexes in species with structurally coloured plumage remain largely unknown. Using full-spectrum spectrometry and transmission electron microscopy, we compared the colour and morphology of rump feathers of male and female eastern bluebirds (*Sialia sialis*). The ultraviolet (UV)-blue feather colour in this species is caused by coherent scattering of light within the medullary 'spongy layer' of feather barbs. This spongy layer lies beneath a keratin cortex and on top of a layer of melanin granules that surround a hollow central vacuole. Irregularly shaped electron-dense regions are present within the cortex. Male and female *S. sialis* differed substantially in their plumage colour and feather structure. A backwards logistic regression predicted sex with 100% accuracy using the colour variables brightness, UV-violet (UV-V) chroma and spectral saturation. A second backwards logistical regression predicted sex with 100% accuracy using relative cortex area and size of air spaces. Thus, *S. sialis* are dimorphic both in colour and in the structures causing this colour. Multiple regression analyses using data pooled from both sexes indicated that multiple features of feather barb structure contributed to colour variation in complex ways. Brightness was negatively related to the relative surface area of cortex in barb cross-sections. Hue was positively related and UV-V chroma was negatively related to the distance between scattering elements (i.e. keratin rods and air spaces) in the spongy layer. In contrast, hue was negatively related and UV-V chroma was positively related to the thickness of the spongy layer. UV-V chroma was also negatively related to the relative area of electron-dense regions in the cortex. Spectral saturation was negatively related to the distance between scatterers and the standard error of the size of air spaces. These results suggest that the dimensions of spongy-layer elements are critical to colour production, but that UV-blue coloration can also be modified by the cortex and the thickness of the spongy layer.

ADDITIONAL KEYWORDS: light scattering – melanin – sexual selection – *Sialia sialis* – structural colour.

INTRODUCTION

Sexual dichromatism in birds is thought to have arisen from a dull monochromatic state through sexual selection favouring conspicuous coloration in males (Darwin, 1871; Wallace, 1889). This view has recently been challenged by a number of studies suggesting that the evolution of dichromatism is considerably more complex than was previously thought (reviewed in Badyaev & Hill, 2003). For example, dichromatism is frequently an ancestral rather than a derived state, and its current expression may be caused by selection for duller plumage in one sex. Genetic drift and indirect selection may also have played roles in creating or maintaining dichromatism (reviewed in Badyaev & Hill, 2003). Thus, a complex suite of factors may have contributed to the striking dichromatism seen in many birds.

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Recent studies have shown that some bird species are dichromatic in both the ultraviolet (UV) and the visible spectra (Andersson & Amundsen, 1997; Keyser & Hill, 1999; Mays *et al*., 2004) or in the UV region alone (Hunt *et al*., 1999; Mahler & Kempenaers, 2002; Eaton & Lanyon, 2003). Because passerine birds can perceive UV wavelengths (Cuthill *et al*., 2000), colours in this range should be considered in studies of the evolution of plumage dichromatism.

The colour of non-iridescent UV or UV-blue feathers is thought to be produced as a function of the size and arrangement of nanostructural elements within the medullary 'spongy layer' of feather barbs (Gadow, 1882; Dyck, 1971a, b; Prum *et al*., 1998, 1999; Prum, Andersson & Torres, 2003). This spongy layer lies beneath a keratin cortex and above a layer of melanin granules that surround a hollow central vacuole. The size and arrangement of keratin rods and air spaces in this layer causes short wavelengths of light to be coherently reflected (Prum, 1999). Small changes in the size or arrangement of these elements can cause substantial variation in the reflected colour (Prum, 1999; Prum *et al*., 2003; Shawkey *et al*., 2003). This type of structural colour is widespread among birds (Dyck, 1976; Prum, 1999), but little is known about the anatomical differences underlying variation in colour display both between sexes and among individuals within a species.

We had two goals in this study. First, we wanted to identify the anatomical basis for sexual dichromatism of UV-blue structural coloration, which, to our knowledge, has never been studied. In doing so, we examined multiple structural elements, including some outside the spongy layer. Most studies of the production of UV-blue plumage colour have focused on the spongy layer (Dyck, 1971a; Prum *et al*., 1998, 1999, 2003; Shawkey *et al*., 2003). However, other elements of feather morphology such as barbule density and features of the cortex may also contribute to colour variation (Finger, 1995; Andersson, 1999). Heavily melanized barbules, for example, may absorb light before it reaches the barb, decreasing the amount of light reflected by the feather. Additionally, the cortex and melanin surrounding the spongy layer may alter the properties of light entering or leaving the barb. Thus, we also included these other elements in our analyses. Second, we wanted to determine whether colour variation could be predicted by nanostructural variation. In a previous study (Shawkey *et al*., 2003), we examined spongy layer structures in relation to individual colour variation in a group of male eastern bluebirds *Sialia sialis*. We demonstrated that the amount of light reflected in the UV-violet (UV-V, 300–420 nm) range was positively related to the number of circular keratin rods and that spectral saturation was negatively related to the standard error of keratin rod diameter. Other colour variables were not predicted, perhaps because we focused on the spongy layer and examined only males. Since males and females exhibit a broader range of colour than do males alone (Gowaty & Plissner, 1998), we pooled data from both sexes with the idea that this extensive variation would help elucidate the relationships between colour and nanostructure.

METHODS

In April 2002, we captured 11 female and nine male *S. sialis* in Lee County, AL (32°35′N, 82°28′W). We removed 8–12 contour feathers from the rump of each bird and stored them in small envelopes in a climate-controlled room until analysis. We taped these feathers in stacks of five, in a manner approximating their natural position on birds (i.e. stacked directly on top of one another), to gloss-free black construction paper and recorded spectral data from them using an Ocean Optics S2000 spectrometer (range 250–880 nm, Dunedin, FL, USA). Using a block sheath that excluded ambient light, we held a bifurcated micron fibre optic probe at a 90° angle 5 mm from the feather surface, creating a measurement area 2 mm in diameter. All data were generated relative to a white standard (WS-1, Ocean Optics). We used OOIbase software to record and average 20 spectra sequentially, and recorded and averaged measurements from five random points on each sample.

From these reflectance spectra, we calculated colour variables for each sample. We restricted these indices to wavelengths of between 300 and 700 nm, as evidence suggests that passerine birds are sensitive to UV wavelengths (300–400 nm; 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 colour reflected by the feathers (e.g. Andersson, Örnborg & Andersson, 1998; Keyser & Hill, 1999, 2000). Brightness, the sum of reflectance from 300 to 700 nm, is a measure of the total amount of light reflected by the feathers (Endler, 1990; Andersson, 1999). UV-V chroma is the percentage of total light reflected in the range 300–420 nm (Andersson *et al.*, 1998). Spectral saturation, the percentage of total light reflected within a range of 50 nm on either side of the hue value, is an index of colour purity (Pryke, Andersson & Lawes, 2001).

Measurement of structural variables

Number of barbules

We attached the proximal rachis of two feathers from each bird to microscope slides using fingernail polish and viewed them at 40× magnification on a dissecting

microscope (Fisher Scientific, Pittsburgh, PA). On each feather, we counted the total number of barbules on the coloured portions of the six distal-most blue barbs on either side of the rachis.

Electron microscopy

All feather barbs from the remaining three feathers were prepared for transmission electron microscopy following the methods of Shawkey *et al*. (2003). Micrographs were taken of each barb ($N = 2$–3 barbs for each bird) at two magnifications: one of the entire barb at 3400×, and one taken of both the cortex and spongy medullary layer at 9100× magnification. All micrographs were taken at the most distal tip of the barb. We took micrographs of a waffle-pattern diffraction grating (Ted Pella, Redding, CA) accurate to 1 nm ± 5% at the same magnifications as the feather micrographs for calibration of the images.

We scanned transmission electron microscopy (TEM) micrograph negatives at 400 dpi using an Epson Perfection 1240 U flatbed scanner, and analysed them using NIH Image v.1.62 (available for download at http://rsb.info.nih.gov/nih-image), the Scanning Probe Image Processor (SPIP) v.2.3207 (Image Metrology, 2002), and SigmaScan Pro v.5.0 (SPSS, 1999).

Microstructural variation

Images at 3400× were imported into NIH Image and calibrated. We measured the total surface area of the anterior portion of the barb, as well as that of the cortex, spongy layer and melanin granules. To calculate the proportion of the barb composed of spongy layer, cortex and melanin, we divided these values by the total surface area of the barb. These values are thus reported as percentage of total barb. To examine the placement of melanin granules, we measured the shortest possible straight-line distance from ten evenly distributed melanin granules to the cortex.

Nanostructural variation

Images were then imported into SPIP (Image Metrology, 2002) and calibrated. To measure variation within the spongy layer, we selected a 600 × 600-pixel section of pure spongy layer from the images at 9100× magnification. This spongy layer is composed of irregularly shaped, as well as more circular, air spaces and keratin rods ('SL' in Fig. 2C). We measured the diameter of all circular keratin rods and air spaces and the mean width of all irregular keratin rods and air spaces within this section using the segment analysis tool of SPIP. The diameters of circular keratin rods and air spaces were not correlated ($r = 0.098$, $P = 0.682$), so we summed the mean diameters of circular keratin rods and air spaces to obtain the distance between scatterers (i.e. the distance between adjacent keratin rods or air spaces, *sensu* Prum *et al*., 2003).

CORTEX

TEM micrographs were imported into Sigma Scan Pro 5.0 and calibrated. To determine if the electron-dense cortical regions (hereafter referred to as EDCRs, Fig. 2C) contributed to scattering or absorption of light, their surface areas were measured throughout each cortical cell in the distal portion of the barb above the spongy layer. The percentage of the cortex's surface area composed of EDCRs was determined by dividing the amount of EDCR surface area per barb by the total surface area measured. The position of the EDCRs was measured as the shortest possible straight-line distance from its distal edge to the outer edge of the cortex using SPIP.

STATISTICAL ANALYSES

All statistical tests were performed using SPSS v.10 (SPSS, 2002). We calculated the mean of each ultrastructural and nanostructural element for each image, and then the mean of all images for each bird.

Selection of variables

To reduce the number of variables for analysis, we selected only those that were likely to be predictive. We thus used one-way ANOVAs to assess differences in colour and structure between sexes, and created correlation matrices for colour and structural variables. When data were not normally distributed, we used Mann–Whitney *U*-tests. In both cases, a threshold significance of $P = 0.05$ was used in the initial selection of variables for further analyses. Only those variables that met this threshold were included in the corresponding regression analysis (Tables 1, 3).

Colour and structural differences between sexes

We then used logistic regressions to determine if sex could be predicted by colour and feather microstructure. In separate tests, sex was the dependent variable and either the selected colour or structural measurements (Table 1) were independent variables. We report all variables that were found to contribute to the overall model for each analysis (Table 2). We used backward selection procedures so that variables that could predict colour in combination with others would be included even if they were not significant themselves (Zar, 1999).

Colour and nanostructure

To test for associations between colour and structural variables, we performed separate multiple linear regressions with colour indices as dependent variables and the selected structural variables listed in Table 3 as independent variables. We used backwards selection procedures for the same reasons as described

Table 1. Comparison of colour and feather structure between male and female *Sialia sialis*

	Males	Females	Sex difference	*F*	*P*
Colour					
Brightness	8047.00 ± 367.90 (13.7)	6522.60 ± 245.10 (12.5)	1524.4	12.68	0.002
UV-V chroma (%)	45.00 ± 1.30 (8.6)	37.30 ± 0.50 (8.6)	7.70	32.21	< 0.001
Spectral saturation (%)	21.20 ± 0.60 (8.9)	18.30 ± 0.03 (6.00)	2.90	27.77	< 0.001
Hue (nm)	401.10 ± 3.60 (2.7)	428.90 ± 3.70 (2.9)	–27.80	17.65	0.001
Structure					
Diameter of circular air spaces (nm)	60.70 ± 1.60 (7.7)	66.80 ± 0.90 (4.3)	–6.10	13.03	0.002
Diameter of circular keratin rods (nm)	59.50 ± 1.50 (7.6)	63.70 ± 0.90 (4.9)	–4.20	5.88	0.026
Standard error of circular air spaces	2.24 ± 0.10 (14.7)	2.58 ± 0.10 (11.6)	–0.04	5.49	0.031
Number of barbules	14.20 ± 13.60 (281)	165.50 ± 65.50 (131.3)	–151.30	U: 19.0	0.020
Distance of spongy layer melanin granules from cortex (μm)	4.90 ± 0.41 (24.8)	3.63 ± 0.24 (22.11)	1.27	7.82	0.012
Proportional cortex area (%)	23.64 ± 0.02 (23.3)	33.90 ± 0.02 (19.2)	–10.26	13.99	0.001
Distance between scatterers (nm)	120.20 ± 1.44 (3.1)	130.50 ± 1.26 (3.7)	–10.30	27.47	< 0.001

Means ± 1 SE, as well as the difference between males and females, are presented. Numbers in parentheses are coefficients of variation. *F*-values are for one-way ANOVAs, unless data were not normally distributed. In these cases, Mann–Whitney *U*-values are shown. Only variables with $P \leq 0.05$ are presented. UV-V, UV-violet.

Table 2. Backwards logistic regression models predicting sex of *Sialia sialis* using colour or feather structure variables

Attribute/ measurement variable	Model log likelihood	Change in –2 log likelihood if term removed	Significance of the change
Colour			
Brightness	–40.63	81.27	< 0.001
UV-V chroma	–31.63	63.25	< 0.001
Spectral saturation	–23.86	47.72	< 0.001
Structure			
Proportional cortex area	–322.19	644.38	< 0.001
Diameter of circular air spaces	–283.14	566.28	< 0.001

Variables used in each test are listed in Table 1. Both overall models were significant (Colour: $\chi^2 = 27.526$, $P = 0.000$; Structure: $\chi^2 = 27.530$, $P = 0.000$), and predicted sex with 100% accuracy. UV-V, UV-violet.

above. Variables that were not normally distributed were $\log_{10}$ transformed.

RESULTS

SPECTROMETRY

Reflectance peaks of males were higher compared with those of females and tended to be more defined (Fig. 1). All colour variables were significantly correlated with one another (Table 3).

ULTRASTRUCTURE

Blue colour was visible on the distal ~3 cm of male barbs, and the distal ~2.5 cm of female ones. Proximal to this blue section, the barb was dull and grey. Barbules were heavily melanized (Fig. 2B), and attached at angles of approximately 45° to the barbs. Adjacent barbs were interlocked by barbules. Thus, the barbules could potentially absorb light entering and leaving the barbs (Fig. 2A). Barbules were distributed bimodally among individuals; birds tended to have

Table 3. Correlation matrix of colour and feather structure variables from *Sialia sialis*

	Brightness	UV-V chroma	Hue	Spectral saturation	Diameter of circular air spaces	Barbules	Distance between scatterers	Proportional cortex area	Standard error of circular air spaces	Proportional surface area of EDCRs	Distance of spongy layer melanin granules from cortex
Brightness	–	0.770**	–0.503*	0.825**	–0.535*	–0.505*	–0.544*	–0.581**	–0.239	0.153	0.325
UV-V chroma	0.770**		–0.802**	0.952**	–0.651**	–0.431	–0.673**	–0.473*	–0.522*	–0.333	0.464*
Hue	–0.503*	–0.802**		–0.668**	0.519*	0.372	0.596**	0.535*	0.496*	–0.045	–0.634**
Spectral saturation	0.825**	0.952**	–0.668**		–0.602**	–0.467*	–0.580**	–0.448*	–0.515*	–0.366	0.378
Diameter of circular air spaces	–0.535*	–0.651**	0.519	–0.602		0.451*	0.775**	0.508*	0.490*	0.123	–0.427
Barbules	–0.505*	–0.431	0.372	–0.467*	0.451*		0.461*	0.378	0.218	–0.142	–0.171
Distance between scatterers	–0.544*	–0.673**	0.596**	–0.580**	0.775**	0.461*		0.499*	0.364	–0.039	–0.396
Proportional cortex area	–0.581**	–0.473*	0.535	–0.448*	0.508*	0.378	0.499*	–	0.324	–0.242	–0.460
Standard error of circular air spaces	–0.239	–0.522*	0.496	–0.515*	0.490*	0.218	0.364	0.324	–	0.424	–0.319
Proportional surface area of EDCRs	–0.153	–0.333	–0.045	–0.366	0.123	–0.142	–0.039	–0.242	0.424	–	0.127
Distance of central melanin granules from cortex	0.325	0.464*	–0.581**	0.378	–0.427	–0.171	–0.396	–0.460*	–0.319	0.127	–

All values are Pearson correlations. *$P \leq 0.05$, **$P \leq 0.01$. Only variables with at least one significant correlation are shown. EDCR, electron dense cortical region; UV-V, UV-violet.

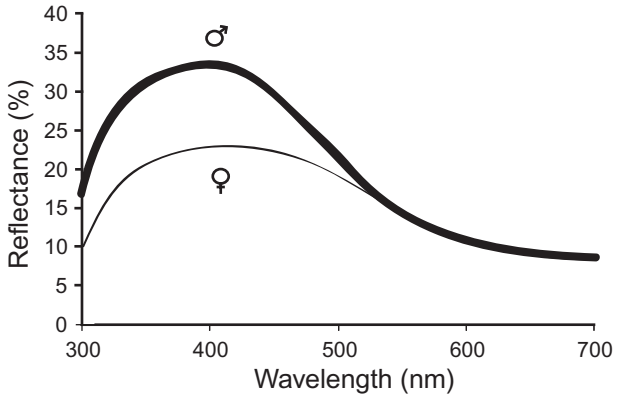


Figure 1. Smoothed average reflectance spectra of male (♂, $N = 9$) and female (♀, $N = 11$) *Sialia sialis* rump feathers.

either many barbules (> 300) or very few. No male had more than 150 barbules, while four of 11 females had over 300 (Fig. 3). In both males and females, the number of barbules increased towards the proximal, dull-coloured ends of barbs.

Nanostructure

As described previously (Shawkey *et al*., 2003), the spongy layer of *S. sialis* feather barbs lies beneath a keratin cortex and above a layer of melanin granules surrounding large central vacuoles (Fig. 2C). The cells of the cortex form several discrete bands and contain irregular EDCRs (Fig. 2C) in most individuals. These electron-dense regions range from small, almost circular shapes to elongate, oblong regions that span throughout cortical cells. Dyck (1971b) speculated that similar areas in the blue feathers of rose-faced lovebirds *Agapornis roseicollis* arose as a result of a unique type of keratinization and noted occasional small clusters of melanin. The density of osmium staining of these EDCRs is similar to that of melanin, but they lack the distinct circular or ovular shape of other melanin granules. Preliminary analyses did not detect the presence of carotenoids (M. D. Shawkey, unpubl. data) in these feathers. Thus, the composition of these dense areas remains uncertain and is the subject of current research. We determined only the surface area of EDCRs within the cortical cells, although occasionally we also saw thickenings of the cortex cell walls. Unlike in the study of Dyck (1971b), cortex density did not vary from the distal to the proximal end. Of 50 micrographs examined, we observed a single melanin granule in the cortex of three.

Although EDCRs can be seen in the cortex of other UV-blue-coloured birds (fig. 4 of Dyck, 1971b; Finger, 1995; Andersson, 1999), these EDCRs have not been investigated thoroughly as far as we are aware. Gower (1936) mentions 'small foreign bodies which appear to have been imbedded in the keratin when it was laid down' that are 'in most cases smaller than the pigment granules' in the cortex of blue jays *Cyanocitta cristata*. Dyck (1971b) mentions EDCRs in the cortex of *A. roseicollis*, but they are restricted to the outer-most cell of the cortex or are thickenings between cortical cells.

Irregularly bent and circular keratin rods and air spaces (Fig. 2C) characterized the spongy layer. These shapes were not distinct structures; rather, they were products of the two-dimensional sectioning of the three-dimensional matrix. Depending on their orientation within the matrix and the angle at which they were cut, rods and air spaces appeared to have different shapes. Measurements of circular elements appear to predict colour more accurately (Shawkey *et al*., 2003) than do those of irregular elements. This type of spongy structure is termed a 'quasi-ordered array' (Prum *et al*., 2003) and is found in several other bird species with UV and UV-blue plumage such as the blue whistling thrush *Myiophonus caeruleus* (Prum *et al*., 2003) and *A. roseicollis* (Prum *et al*., 1999). Previous work has shown that this spongy layer in *S. sialis* is highly organized and able to produce colour by coherent scattering alone (Shawkey *et al*., 2003).

Sexual dichromatism

S. sialis plumage colour was clearly sexually dichromatic (Figs 1, 4, Tables 1, 2). Logistic regression using the variables in Table 1 significantly separated males from females ($\chi^2 = 27.526$, $P = 0.000$) and predicted sex with 100% accuracy (Table 2). Differences in UV-V chroma, brightness and spectral saturation separated the sexes in this model. Male plumage was brighter, more reflective in the UV-V range, and more saturated compared with that of females (Fig. 4A–C).

The feather structure of *S. sialis* is sexually dimorphic. Males and females differed in many aspects of their feather structure (Fig. 5, Tables 1, 2). Backwards logistic regression using the variables listed in Table 1 significantly separated males from females ($\chi^2 = 27.53$, $P = 0.000$) and predicted sex with 100% accuracy (Table 2). Differences in diameter of circular air spaces and relative cortex area separated the sexes in this model (Fig. 5A, B, Table 2).

Colour and structure

When data from both sexes were pooled, between four and six structural variables were significantly correlated with each colour variable (Table 3). Distance between scatterers significantly correlated with every colour variable, suggesting that this is critically important to colour production.

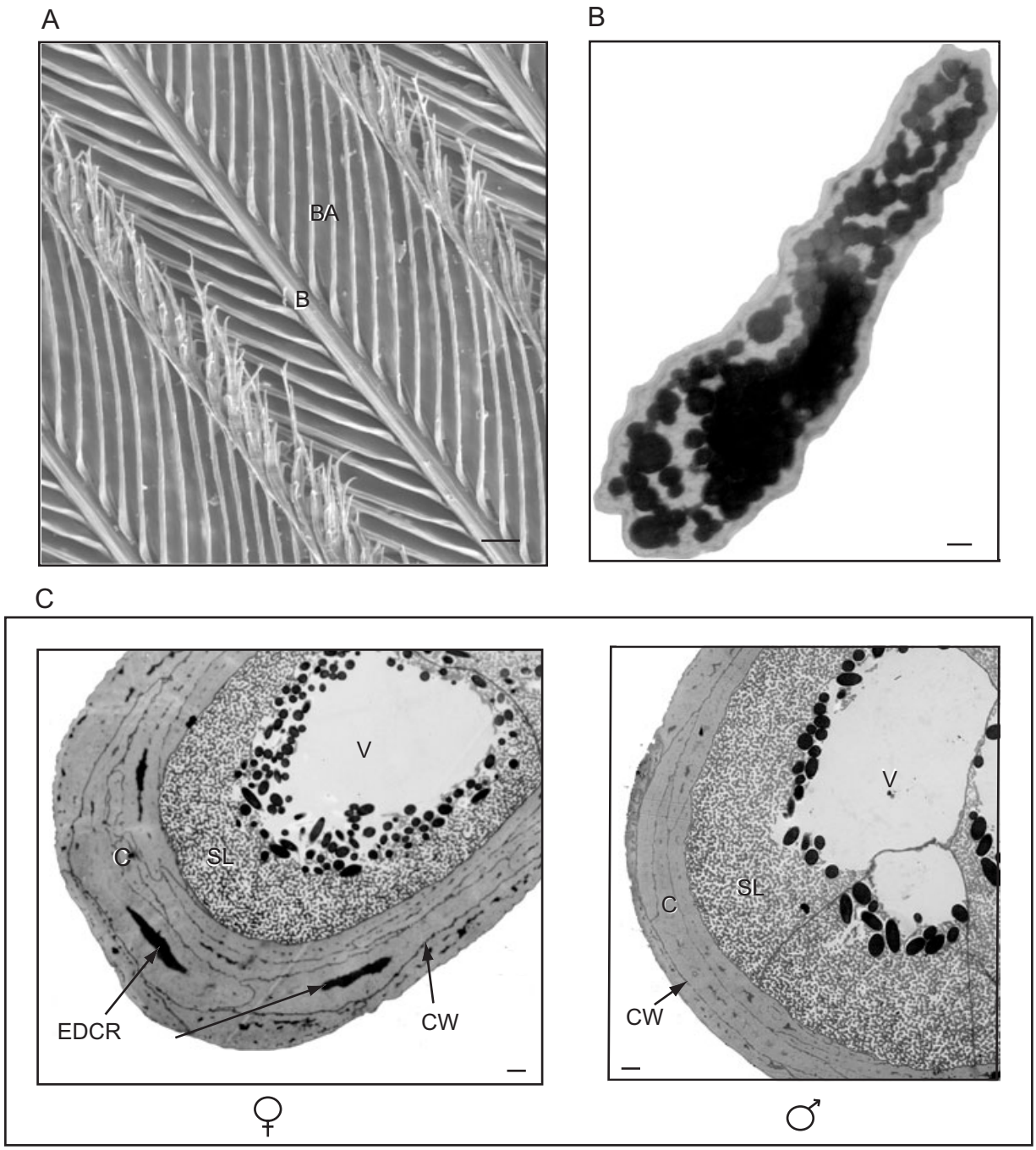


Figure 2. A, SEM (200×) of a *Sialia sialis* feather, showing barbules (= BA) on a barb ramus (= B). Scale bar = 10 μm. B, TEM (3400×) of a cross-section of a barbule from a *S. sialis* rump feather. Electron-dense circles and ovals are melanin granules. Scale bar = 1 μm. C, TEMs (3400×) of cross-sections of male (♂) and female (♀) *S. sialis* feather barbs. c, cortex; EDCR, electron-dense cortical region; CW, cell wall of cortex; SL, spongy layer; V, vacuole. Electron-dense circles and ovals surrounding the vacuole are melanin granules. Scale bars = 1 μm.

Brightness, the total amount of light reflected, decreased as relative cortex area increased (multiple regression: $R^2 = 0.34$, $F_{2,17} = 9.2$, $P = 0.007$; Table 4, Fig. 6). UV-V chroma, the amount of light reflected in the UV-V range, decreased with distance between scatterers and relative area of EDCRs, and increased with distance of central melanin granules from the cortex ($R^2 = 0.65$, $F_{2,17} = 9.9$, $P = 0.001$, Table 4,

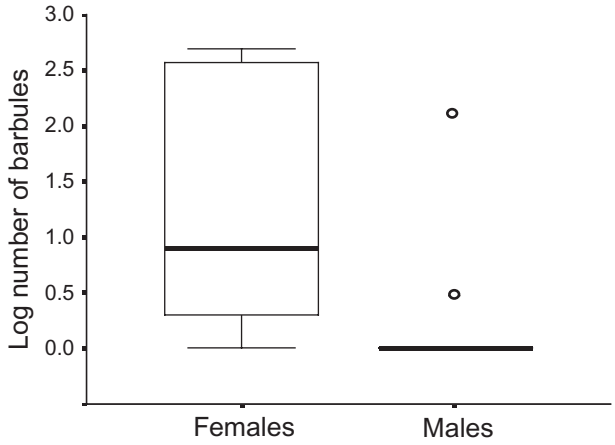


Figure 3. Boxplot of number of barbules/12 barbs ($\log_{10}$ scale) on rump feathers of male and female *Sialia sialis*. The line within each box represents the median number of barbules, the lower and upper borders of each box are the 25th and 75th percentiles and the lower and upper bars are the 10th and 90th percentiles. $N = 11$ females and nine males.

Fig. 7A–C). Hue, the wavelength of peak reflectance, increased with distance between scatterers and distance of central melanin granules from the cortex ($R^2 = 0.47$, $F_{2,17} = 7.5$, $P = 0.005$, Table 4, Fig. 8A, B). Spectral saturation decreased with distance between scatterers and standard error of the diameter of circular air spaces ($R^2 = 0.44$, $F_{2,17} = 6.7$, $P = 0.007$, Table 4, Fig. 9A, B).

These relationships changed when we analysed males and females separately. For females, no element of colour was significantly correlated with any structural variable (all $P > 0.1$). For males, UV-V chroma was negatively correlated with the relative surface area of EDCRs ($r = -0.83$, $P = 0.007$). Hue was correlated with diameter of circular keratin rods ($r = 0.71$, $P = 0.031$) and relative surface area of cortex ($r = -0.666$, $P = 0.050$). Spectral saturation was correlated with relative surface area of EDCRs ($r = -0.72$, $P = 0.026$) and the standard error of diameter of circular keratin rods ($r = -0.72$, $P = 0.030$). Brightness was not correlated with any structural variable. In backwards linear regressions, hue was predicted by diameter of circular keratin rods (multiple regression: $R^2 = 0.51$, $\beta = 0.713$, $F_{2,17} = 7.2$, $P = 0.031$), and spectral saturation was predicted by standard error of circular keratin rods and relative surface area of EDCRs ($R^2 = 0.81$, $\beta_{SE} = -0.554$, $\beta_{EDCR} = -0.569$, $F_{2,17} = 7.2$, $P = 0.007$).

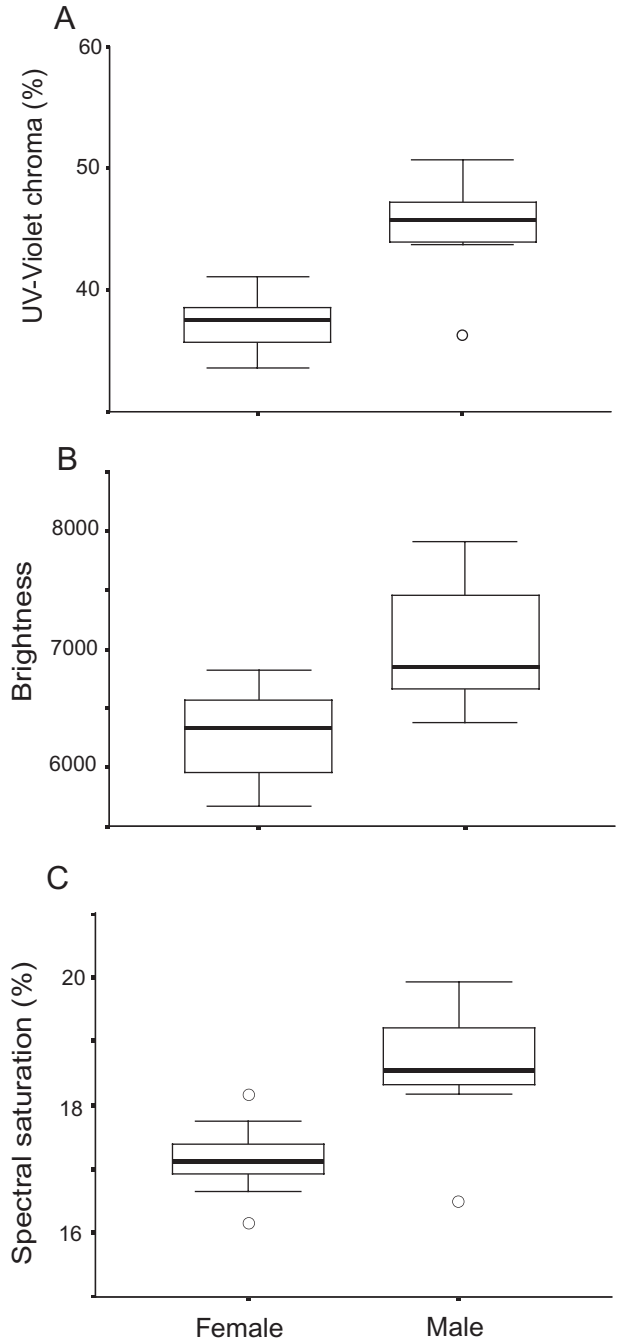


Figure 4. Boxplots of colour variables of *Sialia sialis* feather barbs: A, UV-violet chroma; B, brightness, and C, spectral saturation. The line within each box represents the median colour score, the lower and upper borders of each box are the 25th and 75th percentiles and the lower and upper bars are the 10th and 90th percentiles. $N = 11$ females and nine males.

DISCUSSION

Reflectance spectrometry confirmed that *S. sialis* are sexually dichromatic in both human-visible colour and

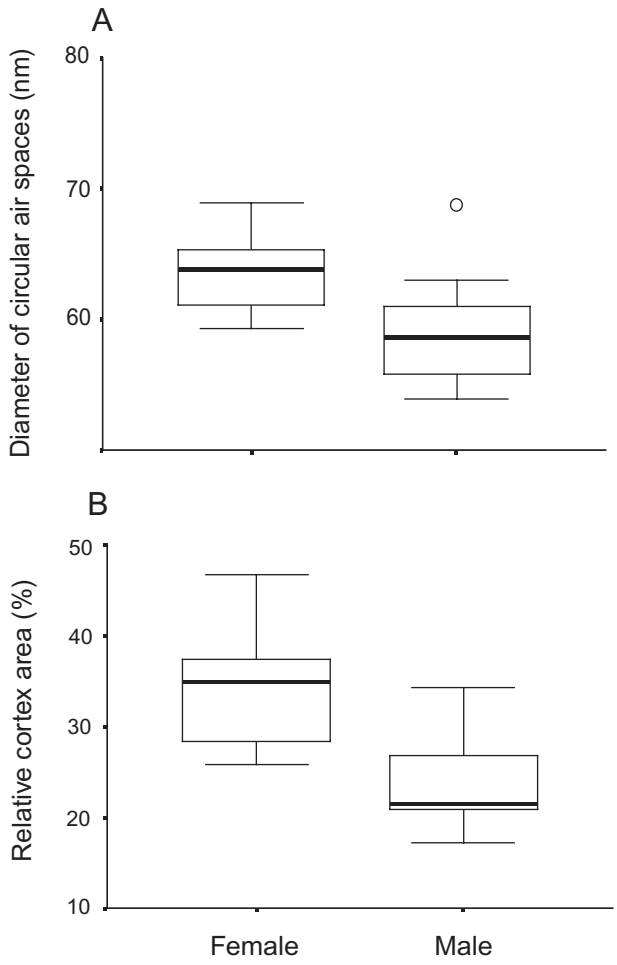


Figure 5. Boxplots of morphological variables in *Sialia sialis* feather barbs: A, diameter of circular air spaces, and B, relative cortex area. The line within each box represents the median colour score, the lower and upper borders of each box are the 25th and 75th percentiles and the lower and upper bars are the 10th and 90th percentiles. $N = 11$ females and nine males.

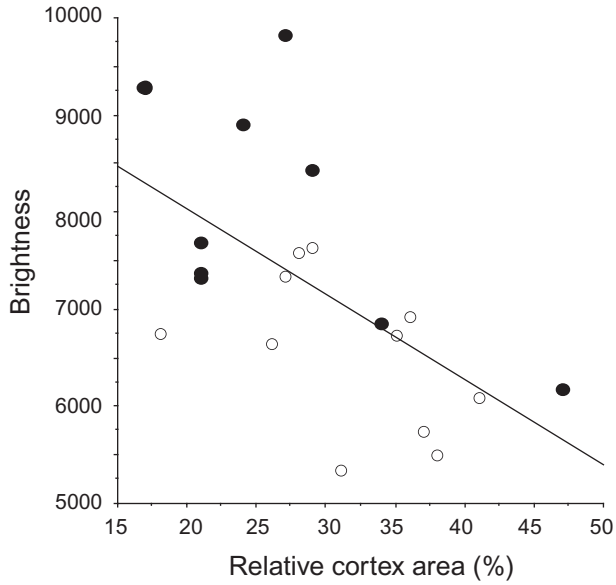


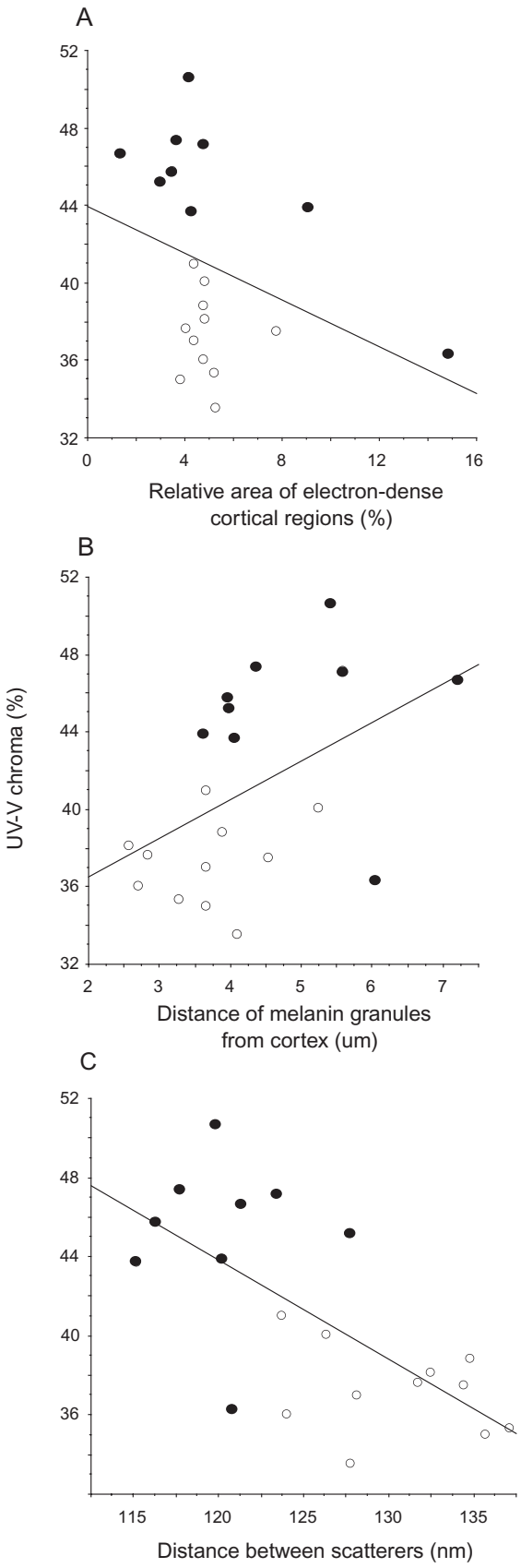
Figure 6. Scatterplot of brightness and relative cortex area of *Sialia sialis* feather barbs. Multiple regression: $R^2 = 0.34$, $F_{2,17} = 9.2$, $P = 0.007$. Filled circles are males, open circles are females. $N = 20$ (11 females and nine males).

the UV range. Indeed, the most striking differences were seen in UV-V chroma (Figs 1, 4). This result is similar to that found by Hunt *et al*. (1999) in the blue tit *Parus caeruleus*, a bird with peak reflectance deeper in the UV region. As many passerines have a retinal UV cone absorbing maximally at 350–380 nm and a blue cone absorbing at 430–455 nm (Bowmaker *et al*., 1997), this result suggests that *S. sialis* are more dichromatic than they appear to human observers. Females reflected maximally at wavelengths about 30 nm higher than did males. However, this difference was apparently not as important as correlated differences in other colour attributes.

Male and female *S. sialis* differed most in the diameter of their circular air spaces and the relative amount of cortex in their barbs. The size of scattering elements such as keratin rods and air spaces in the spongy layer plays a critical role in determining reflected colour (Dyck, 1971a, b; Prum *et al*., 1998, 1999, 2003). Thus, the difference in air space diameter between the sexes may explain many of their colour differences. The thick cortices of female barbs may also decrease the amount of light entering and leaving the barb and thus cause them to appear dull. Over evolutionary time, the reproductive advantage conferred to males with brighter plumage (Siefferman & Hill, 2004) may have caused a reduction in the amount of cortex in their barbs.

The second aim of this study was to describe covariation in structurally based feather colour and nanostructure. Although they were dichromatic, the colour of males and female *S. sialis* lay along a fairly continuous gradient (Figs 6–9). Expanding our previous study to include males and females and examining areas of the feather barb in addition to the spongy layer allowed us to explain much of the variation in every measured aspect of colour.

Brightness was negatively related to the relative amount of cortex in the barb. Using microspectrophotometry, Finger (1995) demonstrated that the cortex absorbs a significant amount of light. It follows that barbs with more cortex will absorb more light. Birds may maximize the signalling properties of their feathers by decreasing the thickness of cortex of their coloured feathers. As the cortex is less porous than the



spongy layer, it is probably important for maintenance of barb integrity and resistance to degradation. Birds may thus trade off structural integrity for signal intensity. Brightness has been shown to play an important role in sexual signalling (Hunt *et al*., 1999), thus this trade-off may be critical to the evolution of structural colour.

UV-V chroma decreased and hue increased with distance between scatterers, while the opposite relationships held for distance from the cortex to the central melanin granules. Spongy layers with larger distances between scatterers will have higher hue values than will those with smaller distances (Dyck, 1971b; Finger, 1995; Prum *et al*., 2003). UV-V chroma may increase with distance to the central melanin granules because more light is scattered and reflected before being absorbed by the melanin and hence more light is coherently scattered into phase. The opposing effects of these factors on hue and UV-V chroma are expected because feathers with hues shifted away from the UV region will necessarily reflect less light in the UV-V range. This reflected light may be further modified by the EDCRs; UV-V chroma decreased with the relative surface area of these regions. Irregularities or substances in the cortex may alter its absorptive properties. While the nature of these regions in the *S. sialis* barb cortex is unclear at present, they may lower UV-V chroma by absorbing light of UV-V wavelengths as it enters or leaves the barb.

Finger (1995) hypothesized that hue values are created by a combination of incoherent scattering in the spongy layer and cortical filtering. This idea was falsified by Prum *et al*.'s (1998) demonstration that hue is created by coherent scattering in the spongy layer alone. This does not, however, rule out a role for the cortex in other aspects of light. Filtering by the overall cortex could cause a generalized decrease in light reflection, while additional filtering by EDCRs could decrease reflection in UV-V wavelengths.

Spectral saturation decreased with distance between scatterers and with variation in the diameter of circular air spaces. Variation in scatterer size thus seems to affect the purity of reflected colour, as has been suggested by some authors (Fitzpatrick, 1998;

Figure 7. Scatterplots of UV-violet (UV-V) chroma and morphological variables in the spongy layer of *Sialia sialis* feather barbs: A, relative surface area of electron-dense regions of the cortex; B, distance of the central melanin granules from the cortex, and C, distance between scatterers in the spongy layer. Multiple regression: $R^2 = 0.65$, $F_{2,17} = 9.9$, $P = 0.001$. (A) $\beta = -0.36$, $P = 0.003$. (B) $\beta = -0.39$, $P = 0.43$. (C) $\beta = 0.29$, $P = 0.098$. Filled circles are males, open circles are females. $N = 20$ (11 females and nine males).

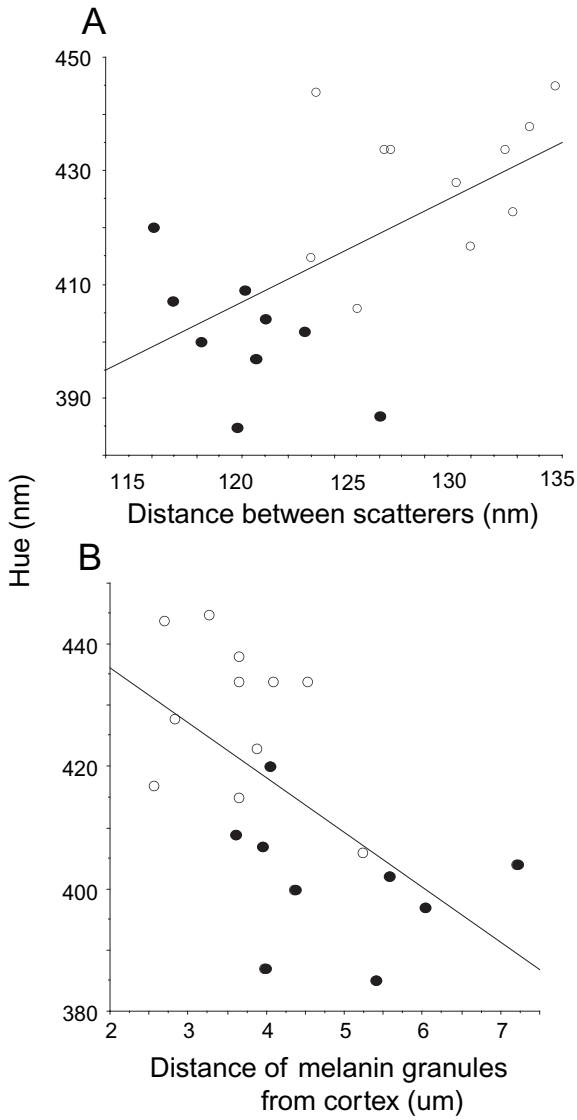


Figure 8. Scatterplots of hue and morphological variables in the spongy layer of *Sialia sialis* feather barbs: A, distance between scatterers in the spongy layer, and B, distance of the central melanin granules from the cortex. Multiple regression: $R^2 = 0.47$, $F_{2,17} = 7.54$, $P = 0.005$. (A) $\beta = 0.43$, $P = 0.034$. (B) $\beta = -0.41$, $P = 0.044$. Filled circles are males, open circles are females. $N = 20$ (11 females and nine males).

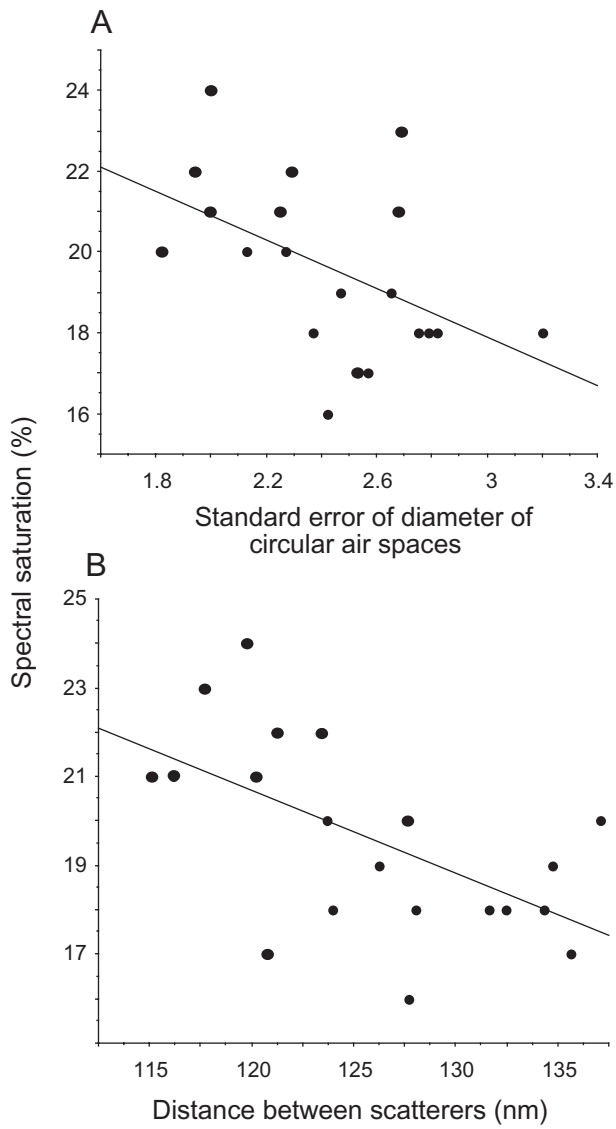


Figure 9. Scatterplots of spectral saturation and morphological variables in the spongy layer of *Sialia sialis* feather barbs. A, standard error of diameter of circular air spaces, and B, distance between scatterers in the spongy layer. Multiple regression: $R^2 = 0.44$, $F_{2,17} = 6.7$, $P = 0.007$. (A) $\beta = -0.45$, $P = 0.033$. (B) $\beta = -0.35$, $P = 0.090$. Filled circles are males, open circles are females. $N = 20$ (11 females and nine males).

Andersson, 1999; Keyser & Hill, 1999). We showed previously (Shawkey *et al*., 2003) that spectral saturation among male *S. sialis* covaries with variation in rod size. However, in this study it covaried with variation in air space diameter. While this seems contradictory, these results may be explained by the overriding importance of distance between scatterers in colour production (Prum *et al*., 1998, 1999, 2003). Variation in the size of either element may have similar effects on reflected colour.

When analysed separately, the patterns of covariation in male barb colour and nanostructure were similar to those of the pooled data, although spectral saturation in males was predicted by variation in size of rods rather than spaces, and also appeared to be affected by the area of EDCRs. This first result is con-

Table 4. Backward linear regression models predicting colour variables using feather structure variables

Dependent variable	Predictors	β	*P*
Brightness	Proportional cortex area	–0.58	0.007
UV-V chroma	Distance between scatterers	–0.36	0.003
	Distance of spongy layer melanin granules from cortex	0.29	0.098
	Relative surface area of EDCRs	–0.39	0.018
Hue	Distance between scatterers	0.43	0.034
	Distance of spongy layer melanin granules from cortex	–0.41	0.044
Spectral saturation	Distance between scatterers	–0.45	0.033
	Standard error of diameter of circular air spaces	–0.35	0.090

Variables used in each test are listed in Table 3. All overall models were significant (Brightness: $r^2 = 0.337$, $F_{2,17} = 9.17$, $P = 0.007$; UV-V chroma: $r^2 = 0.650$, $F_{2,17} = 9.89$ $P = 0.001$; Hue: $r^2 = 0.470$, $F_{2,17} = 7.536$, $P = 0.005$; spectral saturation: $r^2 = 0.442$, $F_{2,17} = 6.74$, $P = 0.007$). UV-V, UV-violet.

sistent with our previous work (Shawkey *et al*., 2003). However, contrary to our previous findings, UV-V chroma was not predicted by number of rods. Either cortical filtering (which we did not account for previously) has a more significant effect than does number of rods, or our sample size in this study was too small to detect its effects. Our inability to explain any variation in female colour may have been caused partially by small sample size, although we explained some variation in male colour with a smaller number of individuals. Either we may have overlooked some aspect of feather structure, or we may need to examine more females to predict colour successfully.

These patterns of covariation are highly suggestive, but they do not provide clear tests of the anatomical mechanisms of colour production by feather barbs. Further experimental work on coloured feathers is needed, particularly on the absorptive properties of cortex and the effects on colour of change in the thickness of the cortex and spongy layer.

We have improved our understanding of the evolution of colourful plumage by elucidating anatomical mechanisms of colour variation. While many studies of quasi-ordered structural colour have focused on the spongy layer (Prum *et al*., 1998, 1999, 2003; Shawkey *et al*., 2003), other aspects of feather structure also contribute to colour variation. The dimensions and arrangement of the spongy layer are clearly critical to the production of colour, but this colour can apparently be modified by the cortex and possibly by other ultrastructural elements such as the barbules. To fully understand the evolution of structural colour on a microevolutionary scale it is necessary to look at several colour variables and aspects of feather structure. The number of studies in which signalling properties of structural colour appear to be based on brightness and/or chroma rather than hue (e.g. Hunt *et al*., 1999; Sheldon *et al*., 1999) make this point clear.

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