FEATHERS AT A FINE SCALE

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FEATHERS AT A FINE SCALE

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DISSERTATION ABSTRACT

FEATHERS AT A FINE SCALE

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Doctor of Philosophy, August 8, 2005
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Directed by Geoffrey E. Hill

In 1668, Antoine von Leeuwenhoek improved the crude microscopes that were being produced in Europe to better study small biological objects (Madigan *et al.* 1997). Although von Leeuwenhoek 's microscope revolutionized biology, helping to support among other things cell theory and germs as the cause of disease, the potential of the microscope escaped the attention of most ornithologists. A small group of researchers has used microscopes to study feathers since the late 19th and early 20th centuries (for historical reviews, see Fox 1976, Prum 1999), but it was not until very recently that a consideration of feathers and the organisms on them has been united with traditional studies of macro colors and structures. The result is a new appreciation of the importance of the bacterial flora of feathers and their potential to serve as a selective force that can affect the colors of feathers.

Microbes were isolated from feathers more than 40 years ago (e.g. Gierløff *et al.* 1961, Pugh and Evans 1970a,b), but the study of feather bacteria remained largely dormant until Burtt and Ichida (1999) isolated feather-degrading *Bacillus* spp. from the feathers of several species. Shawkey *et al.* (2003a) subsequently cultured thirteen distinct isolates from the feathers of house finches (*Carpodacus mexicanus*). More comprehensive surveys using both culture-based and culture-independent methods (see Amann *et al.* 1995 for a review of these methods and their importance in detecting microbial diversity) have revealed even greater microbial diversity on feathers (Shawkey *et al.* in review). While it is now clear that feathers are capable of harboring a diverse microflora, the ecological role(s) of that microflora remain largely a mystery. In this issue of the Auk, Goldstein *et al.* (2004) take us forward in our understanding of those roles.

Using standard microbiological methods, they demonstrated that feather-degrading bacteria degrade unmelanized white feathers more quickly and completely than melanized black feathers *in vitro*. These data, along with those in Burtt and Ichida (in press) suggest that melanin may protect feathers against such bacterial degradation and that many patterns of melanin-based coloration might have evolved in response to bacterial parasitism. Melanized feathers have previously been shown to be harder and more resistant to abrasion than unmelanized feathers (e.g. Burtt 1979, 1986, Bonser 1995, but see Butler and Johnson 2004), but this study is the first to explicitly demonstrate an

enhanced resistance to bacterial degradation in melanized feathers. Melanin-based plumage is used in social signaling (e.g. Rohwer and Rohwer 1978) and may also be involved in thermoregulation (Walsberg 1983) and crypsis (Wallace 1889, Zink and Remsen 1986). The findings of Goldstein *et al.* (2004) suggest that resistance to the degrading effects of bacteria is another important function of melanin, and this observation may have important implications for the evolution of plumage color.

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Introduction

In 1668, Antoine von Leeuwenhoek improved the crude microscopes that were being produced in Europe to better study small biological objects (Madigan *et al.* 1997). Although von Leeuwenhoek 's microscope revolutionized biology, helping to support among other things cell theory and germs as the cause of disease, the potential of the microscope escaped the attention of most ornithologists. A small group of researchers has used microscopes to study feathers since the late 19th and early 20th centuries (for historical reviews, see Fox 1976, Prum 1999), but it was not until very recently that a consideration of feathers and the organisms on them has been united with traditional studies of macro colors and structures. The result is a new appreciation of the importance of the bacterial flora of feathers and their potential to serve as a selective force that can affect the colors of feathers.

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Bacterial degradation of feathers may be an important factor in the evolution of clinal variation in melanin-based color and could be a selective agent responsible for melanic plumage morphs. Burtt and Ichida (in press) suggest that the well-recognized tendency for vertebrates to be more darkly-colored in humid than in arid environments (Gloger's rule) may be partially caused by the better growth conditions for microbes in moister habitats. Song Sparrows (*Melospiza melodia*) living in humid environments showed a consistent trend to have more feather degrading *Bacillus licheniformis* in their plumage (Burtt and Ichida in press) than those from more arid environments. Moreover, the strains of *B. licheniformis* isolated from humid environments degraded feathers more

quickly than those from arid environments under identical lab conditions. Although preliminary, these data suggest that birds in humid environments may be darker because of stronger selection imposed by more potent feather-degrading bacteria.

The work of Burtt and colleagues provides a nice complement to recent work on the genetic basis of melanism in birds. Theron *et al*. (2001) showed that variation in the MC1R locus, a gene coding for a receptor protein involved in melanin synthesis, is perfectly associated with the melanic plumage morph in Bananaquits (*Coereba flaveola*). A single point mutation at this locus causes melanin to be deposited in all feathers, creating a virtually all-black morph. These black morphs are found almost exclusively in moist forests, while yellow morphs are found in dry lowland habitats (Wunderle 1981a,b). Given the observations of Goldstein *et al*. (2004) and Burtt *et al*. (in press), it seems possible that bacterial degradation in moist habitats explains the selective advantage of black morphs there, and hence the retention of the mutant MC1R gene. Mundy *et al*. (2004) show similar associations between the MC1R gene and melanic plumage morphs in Lesser Snow Geese (*Anser c. caerulescens*) and Arctic Skuas (*Stercorarius parasiticus*), as do Doucet *et al*. (in press) in mainland and island populations of White-Winged Fairy-Wrens (*Malurus leucopterus*), but the association between these morphs, habitat humidity, and bacterial degradation is less clear. Bacterial degradation is one of a host of potential selective factors acting on plumage. Among bird species that experience substantial variation in humidity across their range, however, a high percentage adhere to Gloger's rule (~94%, Zink and Remsen 1986), suggesting that bacterial degradation may be important in shaping avian coloration.

Of course, melanin deposition is but one means by which birds color their feathers. Carotenoid pigments are used by many birds to create bright red and yellow colors that tend to be involved in sexual signaling (reviewed in Hill 2002). While the anti-oxidant properties of carotenoids are well studied, their effects on feather structure and potential contribution to degradation resistance are unknown. Other than some microstructural studies of feathers with structural/carotenoid green color (Dyck 1976, Prum *et al*. 1999), the tensile properties, resistance to degradation, and microstructure of feathers with carotenoid color are almost completely unstudied. Such studies would provide great insight into the costs and benefits of having brightly colored plumage.

Much more is known about the anatomy and physical properties of feathers with structurally-based color (for a review, see Prum 1999), which also appears to be used in sexual signaling (Keyser and Hill 1999, Hunt *et al.* 1999, Siefferman and Hill 2004). Structural feather coloration results from one of at least six types of tissues with complex arrangement at a nano-scale (Prum 1999). There is clear potential for interactions between microbes and these microscopic feather structures. In Eastern Bluebirds (*Sialia sialis*) and likely other North American birds with non-iridescent purple and blue coloration, it is the barbs that produce color. Barbs have a central air filled vacuole, a spongy medullary layer composed of a tightly arranged matrix of keratin and air spaces, and a keratin cortex (Shawkey *et al.* 2003b). It is the spongy layer that scatters light in a manner that creates constructive interference with specific wavelengths of light, producing a brilliant color display, and it is this spongy layer that has been the object of most research. There is greater potential for bacterial interaction with the outer keratin cortex than with the spongy layer, and recent evidence suggests that the thickness of the

keratin cortex has a significant effect on brightness (Shawkey *et al*. in press). In Blue Tits (*Parus caeuruleus)* brightness has been shown to increase throughout the breeding season (Örnborg *et al*. 2002). Perhaps bacteria attach to and degrade the keratin cortex, contributing to an overall increase in brightness. Experimental application of feather-degrading bacteria to structurally-colored feathers *in vitro* and *in vivo*, combined with spectrometry and electron microscopy, could be used to test this hypothesis.

Other bird species, particularly those with iridescent color, use structural tissue in their barbules to create color (Prum 1999). The color of reflected light in these species is frequently caused by the layered arrangement of melanin granules beneath a thin keratin cortex (Prum 1999, but see Brink and van der Berg 2004). It is intriguing that color production in barbules, which are much thinner (Lucas and Stettenheim 1972) and hence more susceptible to wear than barbs, is dependent on melanin while color in barbs is not. Indeed, even barbules on colored barbs tend to be heavily melanized. Perhaps this melanization evolved partly as a defense against degradation, and only later became involved in the production of bright color. This question could be addressed through phylogenetic analyses of the mechanisms of structural color.

The potential for new discoveries at the intersection between microbiology and ornithology is enormous. Here Goldstein *et al*. (2004), with a simple experiment, have opened up a realm of possibilities in the entirely new field of evolutionary interactions between microbes and feather color. By using both microscopes and binoculars, we are likely to achieve a better understanding of the function and evolution of feather coloration.

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Chapter 1

Nanostructure predicts intraspecific variation in structural plumage colour.

Summary

Evidence suggests that structural plumage colour can honestly signal quality, but the mechanisms responsible for its variation within a population have not been identified. We used full-spectrum spectrometry and transmission electron microscopy to investigate the effect of variation in spongy layer nanostructure on colour in eastern bluebird (*Sialia sialis*) feathers. Fourier analysis revealed that feather nanostructure was highly organized but did not accurately predict variation in hue. Within the spongy layer of feather barbs, the number of circular keratin rods significantly predicted UV-violet chroma, while the standard error of the diameter of these rods significantly predicted spectral saturation. Thus, the precision of nanostructural arrangement determines some colour variation in feathers, suggesting a potential cost to more intense structural colour.

Introduction

Structural colour is based largely on the size and arrangement of keratin rods and air spaces in the spongy medullary layer of feather barbs (Gadow 1882; Dyck 1971a,b; Prum *et al*. 1998, 1999a). Until recently, it was thought that the reflective properties of spongy tissue were explained by incoherent Rayleigh (Häcker and Mayer 1902) or Mie (Finger 1995) scattering (reviewed in Prum 1999), in which the phase relationships among the scattered lightwaves are random. These models predict that the arrangement of scatterers (i.e. keratin rods and air spaces) is random and that rods of a given size will scatter smaller wavelengths of light more efficiently than larger wavelengths.

Models of coherent scattering, on the other hand, propose that the spongy layer can produce differential propagation of light waves as a result of the interactions among the scattered waves. By this model, the colour observed is the *sum* of the waves scattered by the array, rather than the result of differential scattering of some wavelengths. Dyck (1971a,b), in his 'hollow cylinder model' postulated that the sizes of keratin bars and air spaces in the spongy layer allows constructive interference and coherent scattering of light waves to produce colours. This model was supported by Prum *et al.*'s (1998, 1999a) findings that the spongy layer is not randomly organized and is in fact highly nanostructured and appropriately scaled to produced the observed colours by constructive interference.

Despite substantial interest in the signal content of structural colouration (e.g. Andersson et al. 1997, Bennett et al. 1998, Keyser and Hill 1999), no study has sought associations between colour and nanostructural variables among individuals within a

population. Here we use full-spectrum spectrometry and transmission electron microscopy to investigate the effect of variation in spongy layer nanostructure on colour in eastern bluebird (*Sialia sialis*) feathers. Our aim was to look for correlations between feather microstructure and colouration.

In our investigation, we took a multi-faceted approach. First, we used Fourier analysis to determine if the spongy layer is sufficiently organized to produce colour by coherent scattering and if variation in this organization explains variation in hue. We expected that the spongy layer nanostructure would be highly organized, and that the hues predicted by Fourier analysis of the nanostructure would closely match those measured by the spectrometer. Next, we sought more specific associations between the size and arrangement of structural elements within the spongy layer and colour variables. We predicted that the size or variation in size of these elements would predict measures of hue, brightness, chroma and spectral saturation.

Methods

Sampling

We captured 30 adult male bluebirds in Lee County, Alabama (32°35'N, 82°28'W), USA in June 2002. We pulled 8-12 feathers from the same area on the rump

of each bird and stored them in small manila envelopes in a climate-controlled room until the time of analysis.

Spectrometry

We taped these feathers in stacks to black construction paper and recorded spectral data from them using an Ocean Optics S2000 Spectrometer (range 250-880 nm, Dunedin, Florida, USA) with a micron fibre-optic probe/illuminating device at a 90-degree angle to the feather surface. Ambient light was excluded with a block sheath that held the probe tip at a fixed distance of 5 mm from the feather surface. The reading area of 2 mm diameter of light was illuminated by both a UV (deuterium bulb) and a visible (tungsten-halogen bulb) light source. All reflectance data were generated relative to a white standard (WS-1, Ocean Optics, Dunedin, Florida). We used OOIBase software to record 20 spectra sequentially and average them. The probe was lifted and replaced, and a new measurement taken, five times on each feather sample. We then averaged these five measurements for each sample.

We calculated indices for three different elements of colour from these reflectance spectra (Hailman 1977). These indices were restricted to wavelengths between 300 and 700 nm, as evidence suggests that passerine birds are sensitive to ultraviolet (UV) wavelengths (300-400 nm; Cuthill *et al.* 2000), and that 700 nm is the upper limit of the vertebrate visual system (Jacobs 1981). We measured hue, or the principal colour reflected by the feather, as the wavelength of peak reflectance. This measurement is a commonly used index of spectral location (e.g. Keyser and Hill 1999, 2000; Sheldon *et al.* 1999). Brightness, or the total amount of light reflected by the feather, was measured as the summed reflectance from 300-700 nm (Andersson 1999;

Endler 1990). Chroma, or spectral purity, was measured in two ways. We measured UV-V chroma as the proportion of UV-violet light (i.e. 300-420 nm) reflected. However, this measurement is highly correlated with hue. This correlation is caused by the dependence of the amount of light reflected in the UV on the wavelength of peak reflectance. Feathers with peak reflectance shifted toward the UV will necessarily reflect more light within the range over which UV-V chroma is calculated. To decouple these variables, we also measured "spectral saturation" as the proportion of light reflected within a range of 50 nm on either side of the hue value. From the 30 males for which we had plumage colour data we chose 12 that spanned the range of colour variation in the population.

Microscopy

We cut feather barbs from the upper 1 cm of a contour feather and incubated them in 0.25 M Sodium Hydroxide and 0.1% Tween 20 for 30 min on a bench-top shaker. These barbs were then changed into 2:3 (v/v) of formic acid and ethanol for 2.5 hours. Feather barbs were dehydrated by incubating in 100% ethanol twice and 100% propylene oxide once, and infiltrated with Epon in successive concentrations of 15, 50, 70 and 100%. Barbs were placed into molds with the most distal tip of the barb at the top of the mold, and then the blocks were cured in an oven at 68ºF for 24 hrs. Barbs were cut using a diamond knife on a RMC MT-X (Boeckeler Instruments, Tucson, AZ) ultramicrotome. Sections were placed on a 200 mesh copper grid (EMS, Fort Washington, PA) with formvar support, post-stained in osmium and lead citrate, and viewed on a Phillips EM301 (Veeco FEI Inc, Hillsboro, OR) at 24,000 magnification. All micrographs (n = 3-4 barbs for each bird) of the spongy layer were taken at the most distal tip of the barb,

directly under the cortex, but away from cell margins. We took micrographs of a waffle-pattern diffraction grating (Ted Pella, Redding,CA) accurate to 1 nm ± 5% at the same magnification for calibration of the images.

TEM micrograph negatives were scanned at 400 dpi using an Epson Perfection™ 1240U flatbed scanner, and analyzed using the Scanning Probe Image Processor™ (SPIP) v.2.3.1 (Image Metrology 2002).

Fourier analysis

We analyzed these images using methods of Fourier analysis similar to those of Prum *et al*. (1998, 1999a). We wanted to determine if the nanostructure of bluebird feather barbs was sufficiently organized to produce colour by coherent light scattering, and if variation in the organization of this tissue explained variation in hue (for a more detailed explanation of Fourier analysis, see Prum *et al*., 1999a).

To perform Fourier analysis, micrographs were first calibrated against the waffle-pattern diffraction grating in SPIP. We selected a 1024 x 1024 pixel section of pure spongy layer from these calibrated images, avoiding the cortex and any cell boundaries or melanin granules. To correct for non-linear coupling between the lateral plane and the z-axis in the image, we flattened the image by applying a least mean square plane correction (Image Metrology 2002) to each section. The mean refractive index of each section was estimated using 2-bin histograms of the frequency of dark (air) and light (keratin) pixels and the estimated refractive indices of keratin (RI=1.54) and air (RI=1.00). These values were used in the formula

$$\text{Mean Refractive Index (MRI)} = \frac{(K * 1.54) + (A * 1.00)}{\_\_\_\_\_}$$

where K and A are the percentages of keratin and air in the image, respectively (Dyck 1971b).

The 2D fast Fourier transform (FFT) module of SPIP was used to create 2D Fourier power spectra for each image. The radial spectra were then calculated using the roughness module of SPIP. These radial spectra exhibited two to four discrete peaks varying in strength. We multiplied each peak by twice the MRI for the tissue, resulting in several predicted hue values for each image. Frequently, the value resulting from the strongest peak for each image was outside the visual spectra of vertebrates, likely because of the inherent background "noise" of most biological tissues (J. Jorgensen, pers. comm.). Thus, we selected the value resulting from the strongest peak within the visible spectrum. The values we obtained were close to those obtained from the same images (n = 2) using Prum et al's (1998, 1999a) techniques (R.O. Prum, pers. comm.). We averaged the Fourier-predicted hues from the images for each bird and compared them to those measured using spectrometry.

Nanostructural variation

We measured the diameter of all circular keratin rods and air spaces within the selected area of pure keratin using the segment analysis tool of SPIP. Using this same tool, we measured the mean width of all irregular keratin rods and air spaces. All measurements were performed by one author (M.D.S.). We tested the repeatability of these measurements by performing them three times on all images. Repeatability was high for both irregular (R=0.945 for air spaces, R= 0.930 for keratin rods) and circular

(R=0.855 for air spaces, R= 0.801 for rods) elements. To estimate the diameter of the "hollow cylinders" (circular air spaces surrounded by keratin rods), we added twice the mean width of the irregular keratin bars to the mean diameter of the circular air spaces (Dyck 1971b). We calculated the mean of each nanostructural element for each image, and then the mean of all images for each bird.

Statistical analyses

All analyses were performed on SPSS v.10.0 for Macintosh (SPSS 2001). We performed stepwise linear regressions using number, diameter and standard error of diameter of circular keratin rods and air spaces, diameter of hollow cylinders and mean width and standard error of keratin and air bars as independent variables. Hue, total brightness, UV-V chroma, and spectral saturation were the dependent variables.

Results

Spectrometry

All feathers reflected most strongly in the UV/blue regions of the spectrum (Table 1, Figure 1). Variation was highest in total brightness, and lowest in hue (Table 1). UV-V chroma was significantly negatively correlated with hue (Spearman rank correlation; r_s= -0.902, $p < 0.001$, n =12 samples), and significantly positively correlated with spectral saturation ($r_s = 0.825$, $p = 0.001$, n =12). Spectral saturation was significantly negatively correlated with hue (r_s= -0.741, $p = 0.006$, n =12), such that more UV-reflecting birds tended to have more spectrally pure UV-blue colour. None of the other colour variables were significantly correlated.

Microscopy

The spongy layer of eastern bluebird feather barbs lies beneath a keratin cortex and above a layer of melanin granules surrounding large central vacuoles (Fig 1b). This spongy layer is characterized by irregularly bent, as well as more circular, keratin rods and air spaces (figure 1c, figure 2a). Keratin and air space elements of the spongy layer are close in size (±5nm) to those measured by Finger *et al.* (1992) in the UV-black barbs (hue = 385nm) of black lories *Chalcopsitta atra*. Keratin rods are approximately 30 nm smaller, while air spaces are approximately 15 nm smaller, than those reported by Andersson (1999) for the UV-blue barbs (hue = 340-350 nm) of *Myiophonus caeruleus*.

Fourier analysis

All feather barbs showed discrete rings in the Fourier power spectra (figure 2b), indicating a high degree of uniformity and organization on a nanostructural scale. (Vaezy and Clark 1994; Prum *et al.* 1998, 1999a,b; Briggs and Henson 1995).

The hue values predicted by Fourier analysis and those measured with a spectrometer on the same feathers were not correlated ($r_s = 0.126$, $p = 0.697$, $n = 12$, figure 3), and deviated from one another by values ranging from 10 to 116 nm.

Nanostructural variation

The number of circular keratin rods significantly predicted hue (stepwise linear regression, $\beta = 0.680$, $r^2 = 0.458$, $F_{1,11} = 8.581$, $p = 0.016$), and UV-V chroma ($\beta = -0.677$, $r^2 = 0.462$, $F_{1,11} = 8.466$, $p = 0.016$, figure 4a). Because these two colour variables are not independent, we performed a stepwise linear regression with number of keratin rods as the dependent variable and hue and UV-V chroma as independent variables. Only UV-V

chroma was significantly predicted by the number of keratin rods ($r^2 = 0.462$, $p = 0.016$). The standard error of the diameter of circular keratin rods significantly predicted spectral saturation ($\beta = -0.706$, $r^2 = 0.498$, $F_{1,11} = 9.930$, $p = 0.010$, figure 4b). No variable significantly predicted total brightness.

Discussion

The discrete rings in the Fourier power spectra demonstrate that colour production in this species is caused primarily by coherent light scattering due to nanostructural arrangement of keratin and air in the feather barbs (Prum *et al.* 1998, 1999a,b). These rings are comparable to those found in other ordered tissue such as the caruncles of malagasay asities (Prum *et al.* 1999b), the human sclera (Vaezy and Clark 1994), and other structurally coloured feather barbs (Prum 1998, 1999a), and show that the nanostructure is uniform in all directions. Fourier analysis allowed us to confirm that the nanostructure of bluebird feathers is ordered, but it did not successfully predict the hue of individual birds.

Fourier analysis appears to lack the resolution to accurately predict the small-scale variation we observed. Previously reported Fourier-predicted hue values have been from 0-75 nm away from measured values for feather barbs (Prum *et al*. 1998, 1999a), and up to 117 nm away for other tissues (Prum *et al*. 1999b). Resolution could be improved and error could be reduced by increasing the number of micrographs examined for each barb (Prum *et al*. 1999b; R.O. Prum pers. comm.), but Fourier analysis is

probably more useful for comparing large-scale differences in colour between species (e.g. Prum *et al.* 1998, 1999a) than small-scale differences within species.

Although our efforts to predict intraspecific variation in hue using Fourier analysis were unsuccessful, we found that morphological variables within the spongy layer predicted variation in colour quite well. The standard error of the diameter of circular keratin rods strongly predicted spectral saturation, while the number of circular keratin rods strongly predicted UV-V chroma. The negative relationship between the amount of variation in the diameter of keratin rods and the purity of the reflected colour suggests that the precision of the nanostructural elements determines spectral saturation, as has been speculated by some authors (Andersson 1999; Fitzpatrick 1998; Keyser and Hill 1999). When elements are uniform in size, the reflected light will be tightly grouped around the wavelength of peak reflectance. Increasing the variation in those elements may break up this tight grouping and spread the reflected light over a broader spectrum. To our knowledge, this is the first demonstration of possible mechanisms for intraspecific variation in structural colour.

Both coherent (Benedek 1971) and incoherent (Kerker *et al.* 1966; Kerker 1969; Finger 1995) models of colour production predict positive relationships between number of scatterering elements and intensity (brightness) of the reflected light. The number of circular keratin rods in our study predicted relative brightness in the UV-V range, but not overall brightness. This effect could be restricted to this range because other factors such as thickness of the keratin cortex alter total brightness (see below). In any case, the highly ordered nanostructure of these barbs argues strongly against Rayleigh and Mie

scattering as the basis of colour production. Models of coherent scattering are more likely to explain this relationship.

Other colour variables were not significantly related to the structural elements of feathers that we measured. Between species, hue is generally positively associated with the size of keratin and/or air elements (Dyck 1971b; Dyck 1976; Finger *et al*. 1992; Finger 1995). In our comparisons of male eastern bluebirds, hue was not positively associated with the size of any structural elements. However, hue was the least variant of our colour measurements (see Table 1). We may need larger sample sizes to discern any effect of structural element size on hue. The relationship between hue and number of keratin rods appears to be caused by the tight correlation between hue and UV-V chroma.

Variation in total brightness was not explained by our data, and may be caused by morphological features outside of the spongy layer. A layer of melanin granules lies beneath the spongy layer of bluebirds and is needed to prevent backscattering from underlying feather structures (Prum 1999). Melanin granules were present in the spongy layer of one male with low brightness in this study, and in several uniformly dull females (pers. obs.). The presence of melanin granules in the spongy layer could reduce the surface area available for reflection. The thickness of the keratin cortex overlying the spongy layer may also vary between males. A thicker cortex could filter more light entering and leaving the spongy layer than a thin cortex and could thereby decrease brightness (Andersson 1999; Finger 1995). Finally, the heavily melanized barbules may cause a decrease in overall brightness of the feather barbs (Andersson 1999), and duller males may have more of these barbules. As brightness has been shown to play an

important role in sexual signaling (Hunt *et al*. 1999; Doucet and Montgomerie in press), understanding the mechanisms by which it varies should be a high priority for future research.

Recent work suggests that structural plumage colour may serve as an honest, sexually selected signal (Andersson et al. 1997, Bennett et al. 1998, Keyser and Hill 1999; Sheldon *et al*. 1999; Siefferman and Hill *in press*). We have shown that the precision of structural elements determines the spectral saturation of colour. These results suggest that the honesty of structural signals may be maintained by the cost of producing them (handicap model, Zahavi 1975). Alternatively, the tightly constrained physiological processes involved in their production may make them necessarily 'honest' (revealing indicator model; Iwasa et al. 1991). Future work should examine whether expression of the nanostructure responsible for bright structural plumage characteristics is energetically costly and hence limited to those in excellent condition during feather growth. Such tests will provide great insight into the evolution of these traits.

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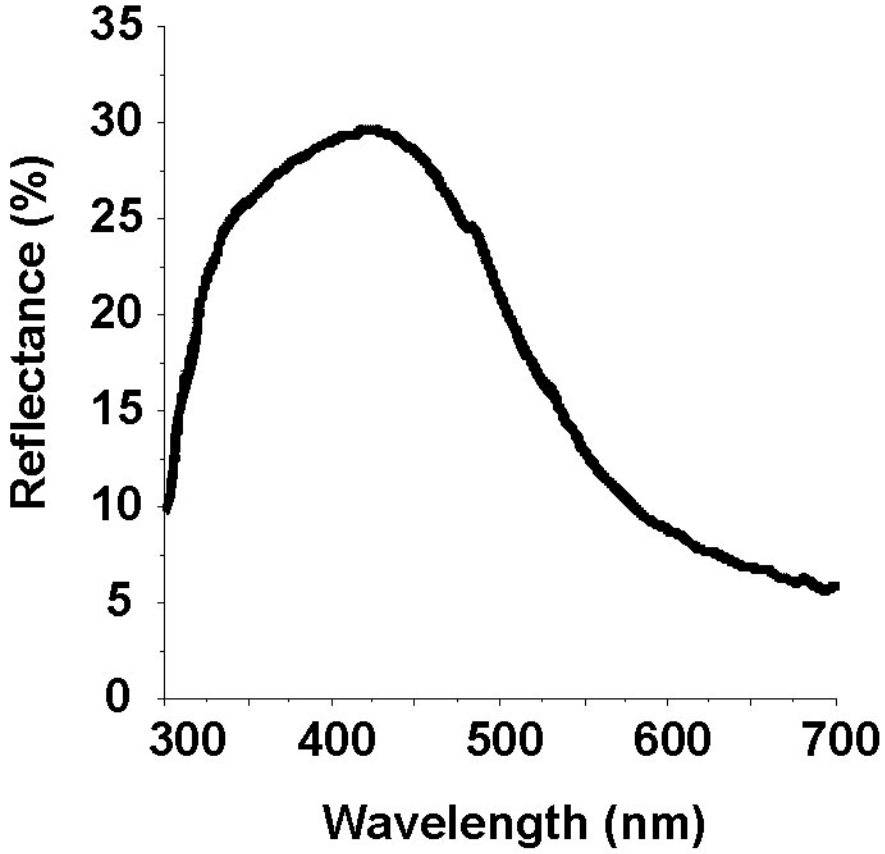
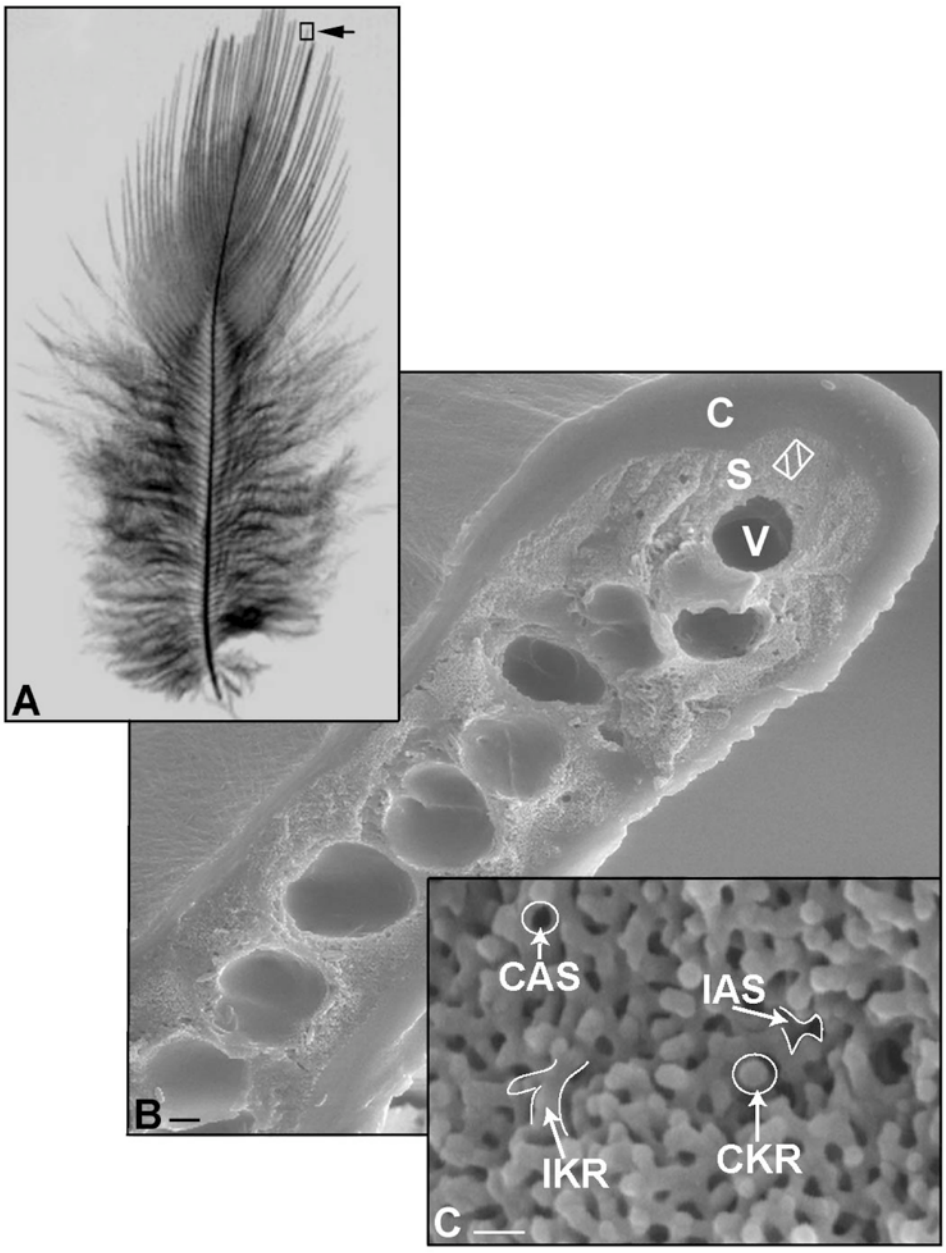
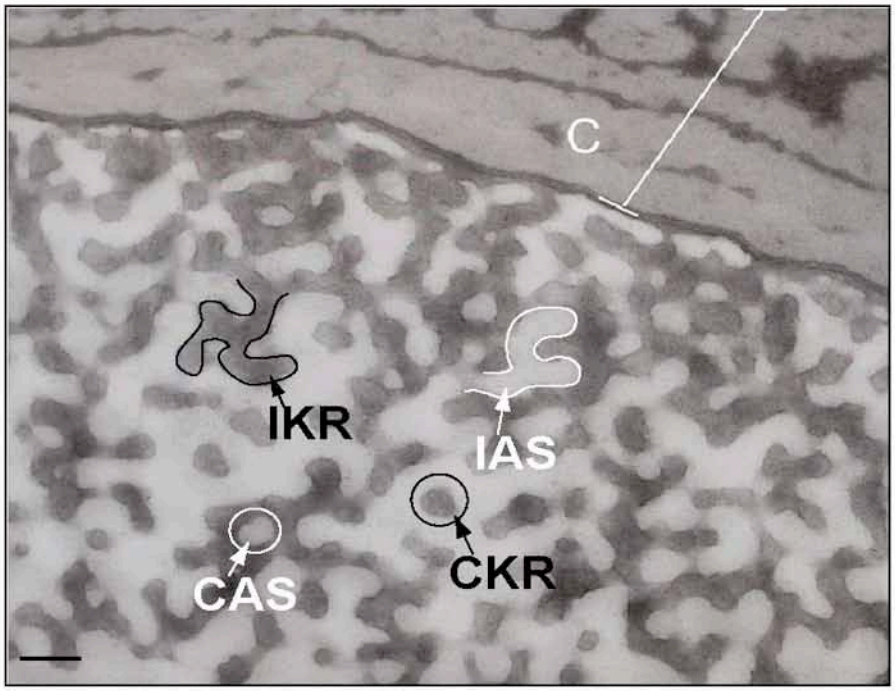
Table 1. Means, standard deviations, and coefficients of variation of color variables and microstructural elements of eastern bluebird feather barb spongy layer.

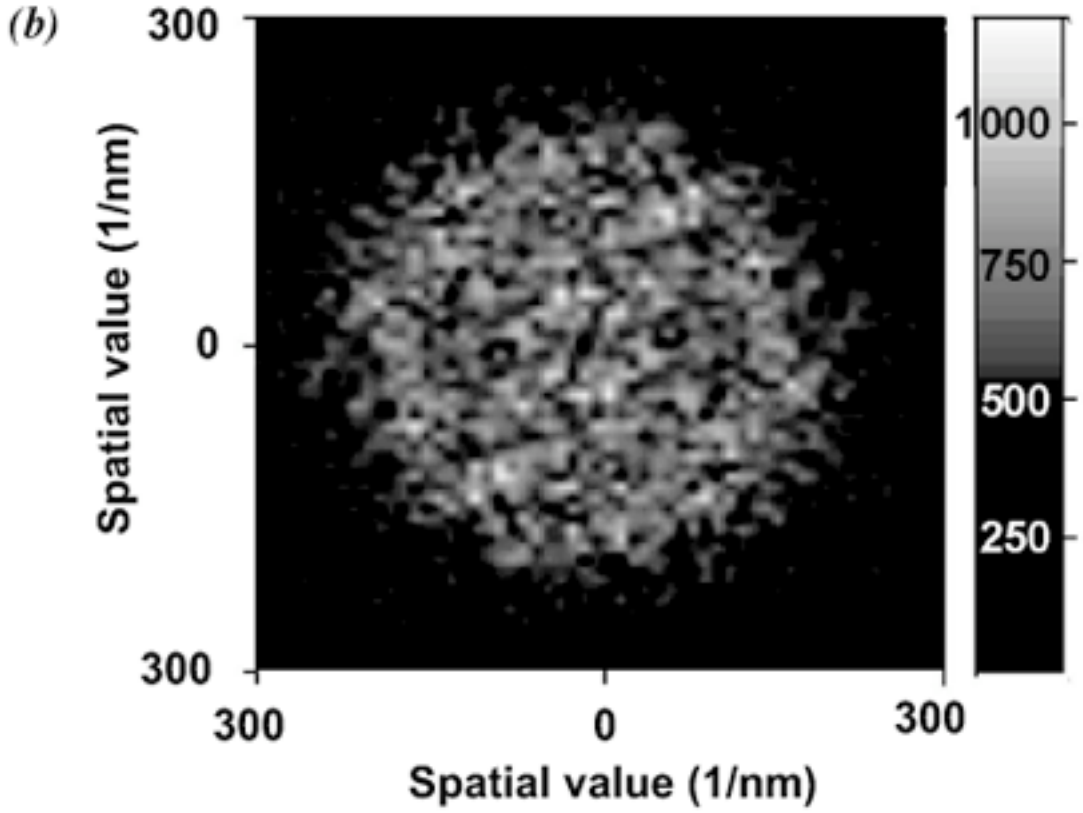
Variable	Mean	±1 SD	CV
Hue (nm)	410.80	8.65	2.10
Mean brightness	20.53	2.81	13.68
UV- violet chroma (%)	0.44	.02	4.54
Keratin circle diameter (nm)	47.88	5.71	11.93
Number of keratin circles	32.17	10.8	33.57
Air circle diameter (nm)	51.86	5.55	10.70
Number of air circles	31.5	8.57	27.21
Mean width of keratin bars (nm)	45.47	4.67	10.27
Number of keratin bars	3	0.15	5.00
Mean width of air bars (nm)	59.21	7.93	13.40
Number of air bars	11	0.85	7.27

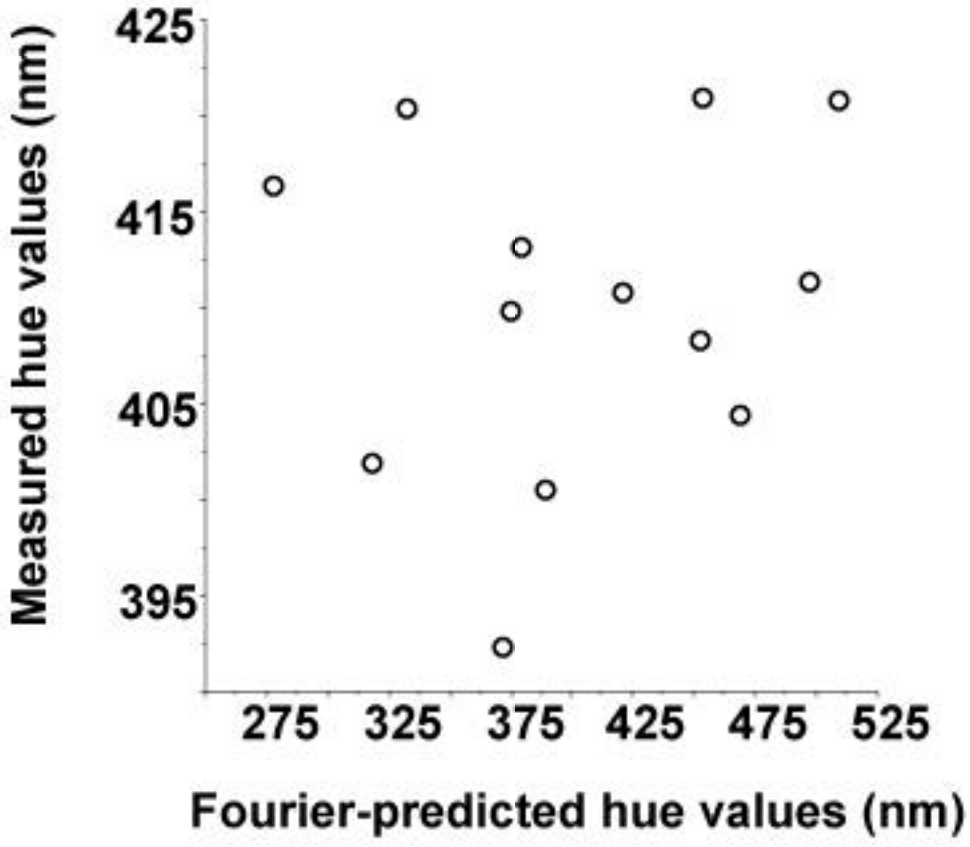

35
30
25
20
15
10
5
0
Reflectance (%)
300
400
500
600
700
Wavelength (nm)

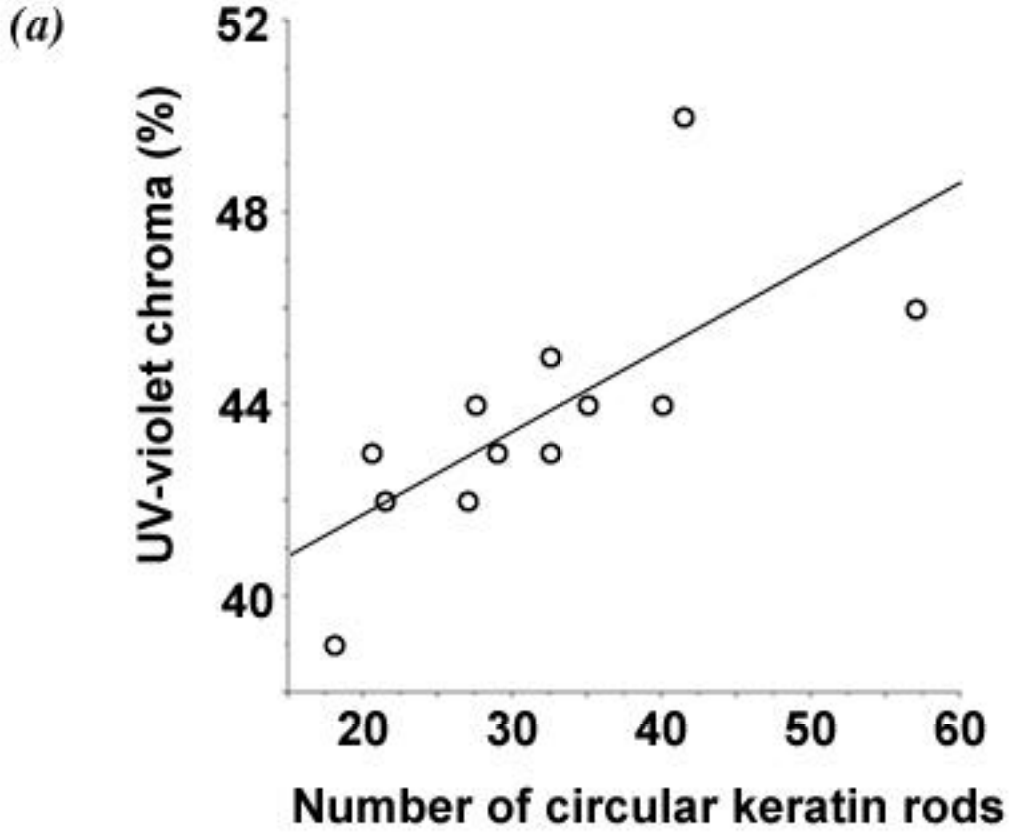

A
B
C
S
V
CAS
IAS
IKR
CKR

A

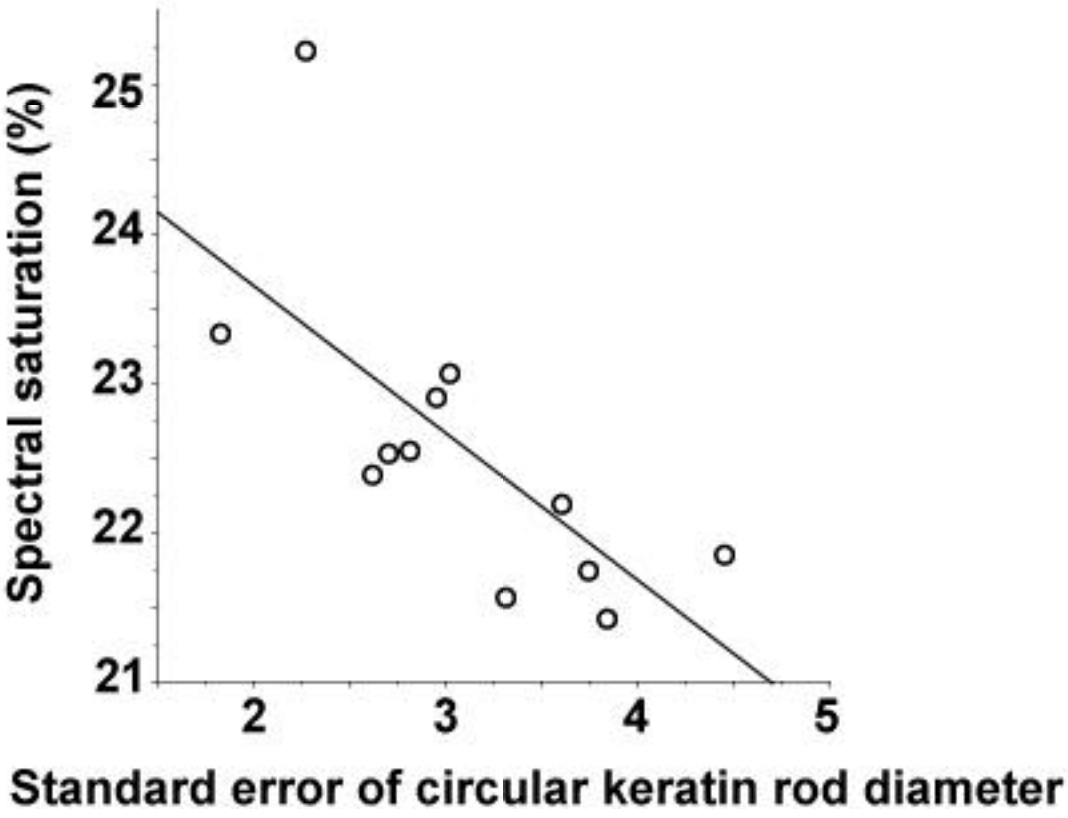



(b)
300
0
300
Spatial value (1/nm)
300
0
300
Spatial value (1/nm)
1000
750
500
250

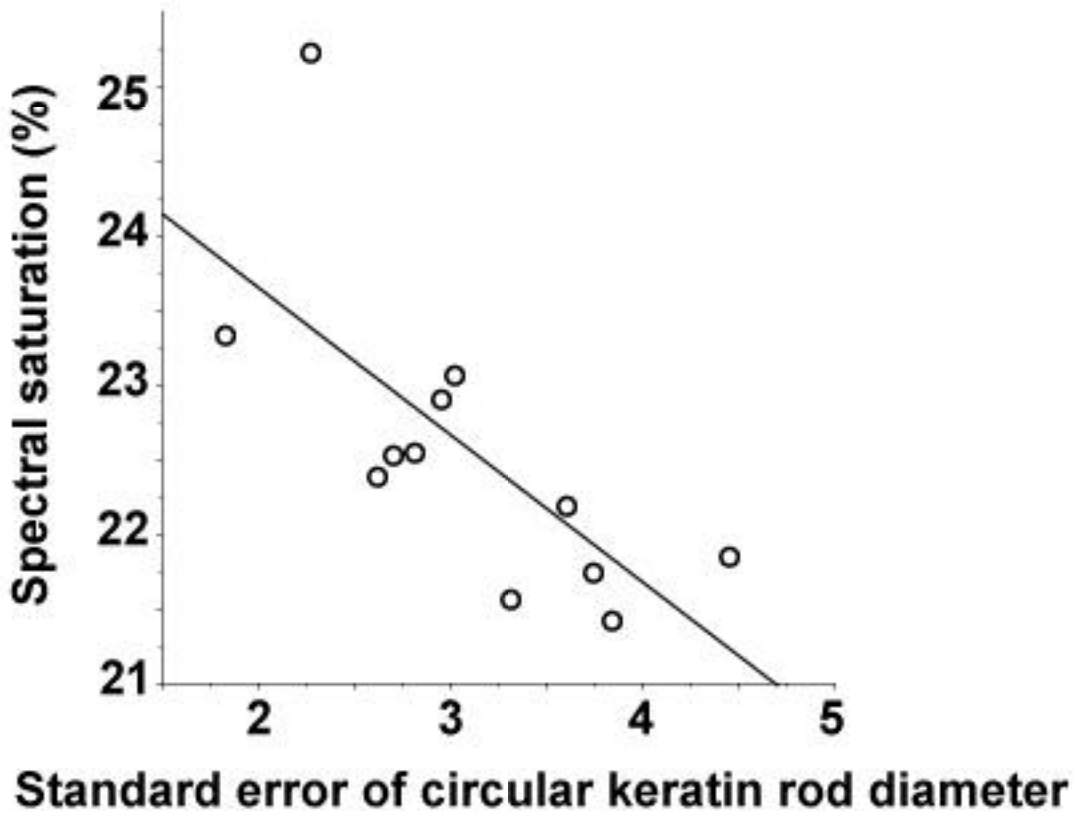

425
415
405
395
Measured hue values (nm)
275
325
375
425
475
525
Fourier-predicted hue values (nm)


(a)
52
48
44
40
UV-violet chroma (%)
20
30
40
50
60
Number of circular keratin rods

(b)


Spectral saturation (%)
25
24
23
22
21
2
3
4
5
Standard error of circular keratin rod diameter

(b)


Spectral saturation (%)
25
24
23
22
21
2
3
4
5
Standard error of circular keratin rod diameter

Chapter 2

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

Abstract

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*). Ultraviolet-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 bluebirds differed substantially in their plumage colour and feather structure. A backwards logistic regression predicted sex with 100% accuracy using the colour variables brightness, 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, bluebirds 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 ultraviolet-violet 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 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 ultraviolet-blue colouration can also be modified by the cortex and the thickness of the spongy layer.

Introduction

Sexual dichromatism in birds is thought to have arisen from a dull monochromatic state through sexual selection favoring conspicuous colouration 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 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.

Recent studies have shown that some bird species are dichromatic in both the ultraviolet (UV) and visible spectrum (Andersson & Amundsen 1997; Keyser & Hill, 1999; Mays *et al*. 2004) or in the UV 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 UV or UV-blue feathers is thought to be commonly produced as a function of the size and arrangement of nanostructural elements within the medullary "spongy layer" of feather barbs (Gadow, 1882; Dyck, 1971 a,b; Prum *et al*.,1998, 1999, 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 & Estes *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 ultraviolet-blue structural colouration, 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 & Estes *et al.*, 2003). However, other elements of feather morphology such as barbule density and features of the cortex may also contribute to colour variation (Andersson, 1999; Finger, 1995). 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 include 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 & Estes *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 ultraviolet-violet (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 only examined males. Since males and females exhibit a broader range of colour than 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 9 male bluebirds 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 fiber optic probe at a 90° angle 5mm from the feather surface, creating a measurement area of 2mm 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 between 300 and 700 nm, as evidence suggests that passerine birds are sensitive to ultraviolet (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, 1998, Keyser & Hill, 1999, 2000). Brightness, the sum of reflectance from 300-700 nm, is a measure of the total amount of light reflected by the feathers (Andersson, 1999; Endler, 1990). UV-violet (UV-V) chroma is the percentage of total light reflected in the range of 300-420 nm (Andersson, Örnborg & Andersson, 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 40x 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 and Estes *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 3,400x, and one micrograph taken of both the cortex and spongy medullary layer at 9,100x 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 of the feather micrographs for calibration of the images.

We scanned TEM micrograph negatives at 400 dpi using an Epson Perfection™ 1240U flatbed scanner, and analyzed 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 3,400x were imported into NIH Image and calibrated. We measured the total surface area of the anterior portion of the barb, as well as 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 10 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 x 600 pixel section of pure spongy layer from images at 9,100x magnification. This spongy layer is composed of irregularly-shaped, as well as more circular air spaces and keratin rods (see "SL" of figure 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, see figure 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 in 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 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 $p \leq 0.05$ 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 (see 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 (see Table 1) were independent variables. We report all variables that 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 above. Variables that were not normally distributed were $\log_{10}$ transformed.

Results

Spectrometry

Reflectance peaks of males were higher than those of females and tended to be more defined (Figure 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 females. Proximal to this blue section, the barb is dull and grey. Barbules were heavily melanized (see figure 2b), and attached at approximately 45 degree angles to the

barbs. Adjacent barbs were interlocked by barbules. Thus, the barbules could potentially absorb light entering and leaving the barbs (see figure 2a). Barbules were distributed bimodally among individuals; birds tended to either have many barbules (>300) or very few. No male had more than 150 barbules, while 4 of 11 females had over 300 (figure 3). In both males and females, number of barbules increased toward the proximal, dull coloured ends of barbs.

Nanostructure

As described previously (Shawkey & Estes *et al*., 2003), the spongy layer of eastern bluebird feather barbs lies beneath a keratin cortex and above a layer of melanin granules surrounding large central vacuoles (Figure 2c). The cells of the cortex form several discrete bands and contain irregular EDCRs (see figure 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 have not detected the presence of carotenoids (M.D. Shawkey, unpublished data) in these feathers. Thus, the composition of these dense areas remains uncertain and is the subject of current research. We only determined the surface area of EDCRs within the cortical cells, although we also occasionally saw thickenings of the cortex cell walls. Unlike in Dyck (1971b), cortex density did not vary from the distal to 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 (see Figure 4 of Andersson, 1999; Dyck, 1971b; Finger, 1995), 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 the rose-faced lovebird; however, 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) characterize the spongy layer. These shapes are not distinct structures; rather, they are products of the two-dimensional sectioning of the three-dimensional matrix. Depending on their orientation within the matrix and the angle at which they are cut, rods and air spaces will appear to be different shapes. Measurements of circular elements appear to predict colour more accurately (Shawkey & Estes *et al.* 2003) than 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 the rose-faced lovebird (Prum *et al.,* 1999). Previous work has shown that this spongy layer in bluebirds is highly organized and able to produce colour by coherent scattering alone (Shawkey & Estes *et al.*, 2003).

Sexual dichromatism

Bluebird plumage colour is clearly sexually dichromatic (Figures 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 than that of females (figure 4a-c).

The feather structure of bluebirds is sexually dimorphic. Males and females differed in many aspects of their feather structure (see Figure 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 (Figure 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 (see table 3). Distance between scatterers significantly correlated with every colour variable, suggesting that it is critically important to colour production.

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, figure 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 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, figure 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, figure 9a-b).

These relationships changed when we analyzed 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 ($R^2 = 0.81$, $\beta_{SE} =-0.554$, $\beta_{EDCR} = -0.569$, $F_{2,17} = 7.2$, $p = 0.007$).

Discussion

Reflectance spectrometry confirmed that bluebirds are sexually dichromatic in both human-visible colour and the ultraviolet range. Indeed, the most striking differences were seen in UV-V chroma (see figures 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. As many passerines have a retinal UV cone absorbing maximally at 350-380 and a blue cone absorbing at 430-455 (Bowmaker *et al.*,1997), this result suggests that bluebirds are more dichromatic than they appear to human observers. Females reflected

maximally at wavelengths about 30 nm higher than males. However, this difference was apparently not as important as correlated differences in other colour attributes.

Male and female bluebirds differ 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, 1971 a,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 are dichromatic, the colour of males and female bluebirds lies along a fairly continuous gradient (see figures 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 signaling 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 signaling (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 those with smaller distances (Prum *et al.*, 2003; Finger, 1995; Dyck, 1971b). 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 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 bluebird barb cortex is unclear at present, they may lower UV-V chroma by absorbing light in 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 (Andersson, 1999; Fitzpatrick, 1998; Keyser & Hill, 1999). In previous work (Shawkey & Estes *et al.,* 2003), we showed that spectral saturation among male bluebirds covaried with variation in rod size. However, in the present work it covaries 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,b, 2003). Variation in the size of either element may have similar effects on reflected colour.

When analyzed 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 consistent with our previous work (Shawkey & Estes *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 previously account for) has a more significant effect than 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. We may

have either overlooked some aspect of feather structure, or we may need to examine more females to successfully predict colour.

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 of change in the thickness of the cortex and spongy layer on colour.

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 & Estes *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 signaling 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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Table 1. Comparison of colour and feather structure between male and female eastern bluebirds (*Sialia sialis*). Means ± 1 S.E., 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.

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

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

Attribute	Measurement variable	Model log likelihood	Change in -2 log likelihood if term removed	Significance c 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

Table 3: Correlation matrix of colour and feather structure variables from eastern bluebirds (*Sialia sialis*). All values are Pearson correlations. *P≤0.05, **P≤ 0.01. Only variables with at least one significant correlation are shown.

	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	0.325	0.464*	-0.581**	0.378	-0.427	-0.171	-0.396	-0.460*	-0.319	0.127	----------

Table 4: Backward linear regression models predicting colour variables using feather structure variables. 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: $r^2 = 0.442$, $F_{2,17} = 6.74$, $p = 0.007$).

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

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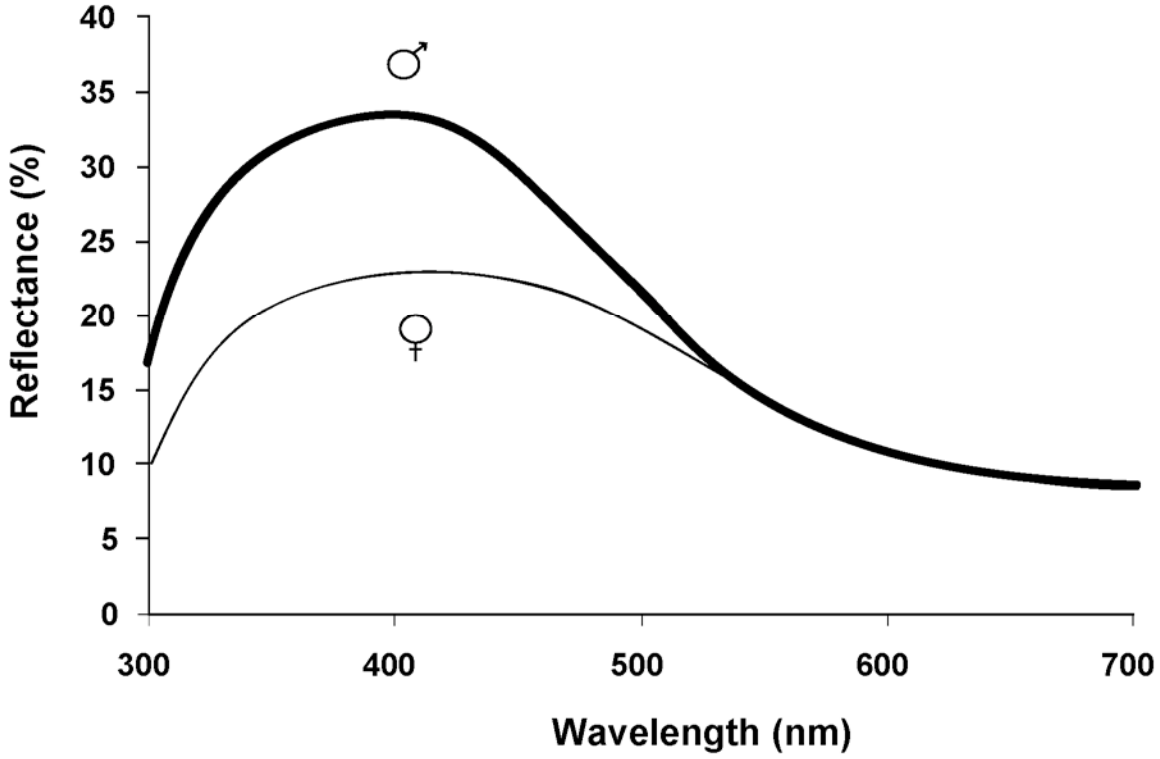
40
35
30
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Reflectance (%)
♂
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300
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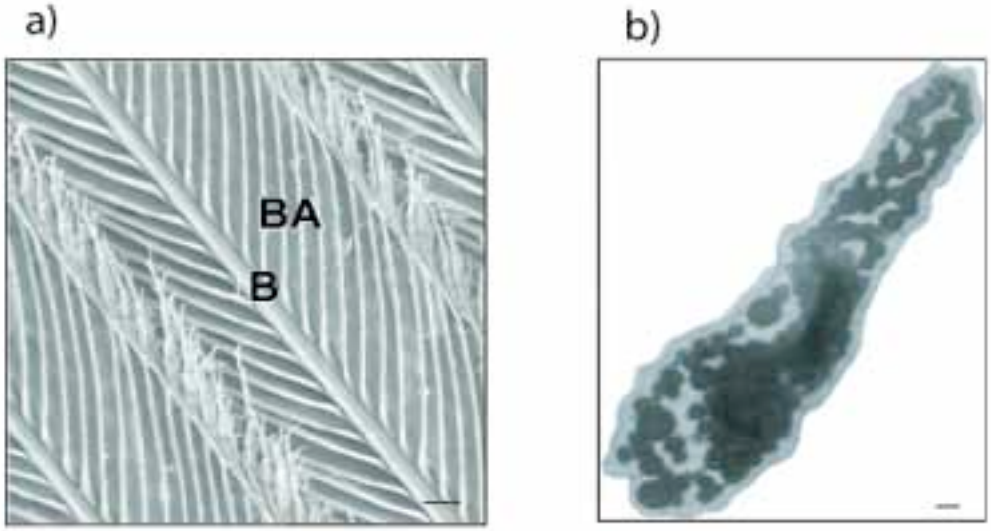
Wavelength (nm)


a)
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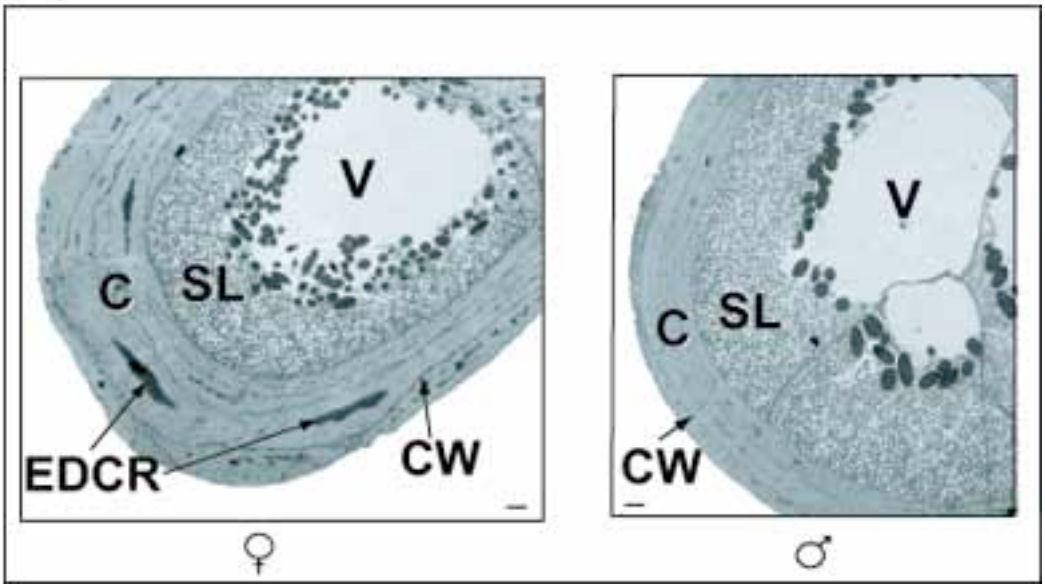
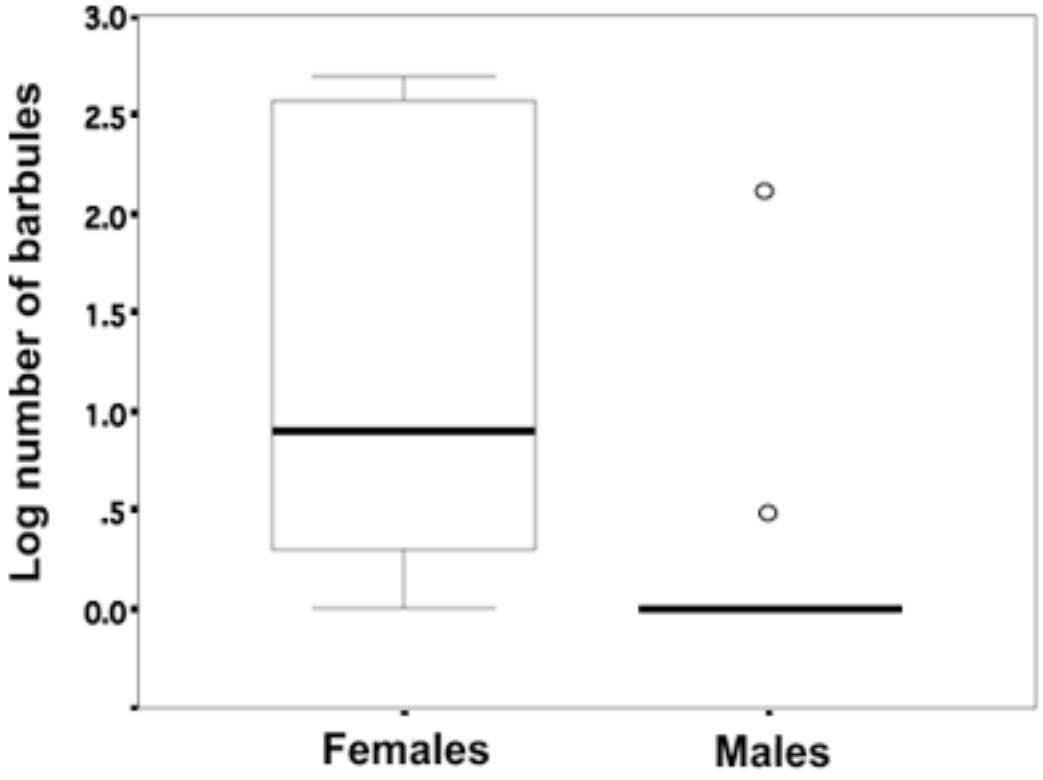
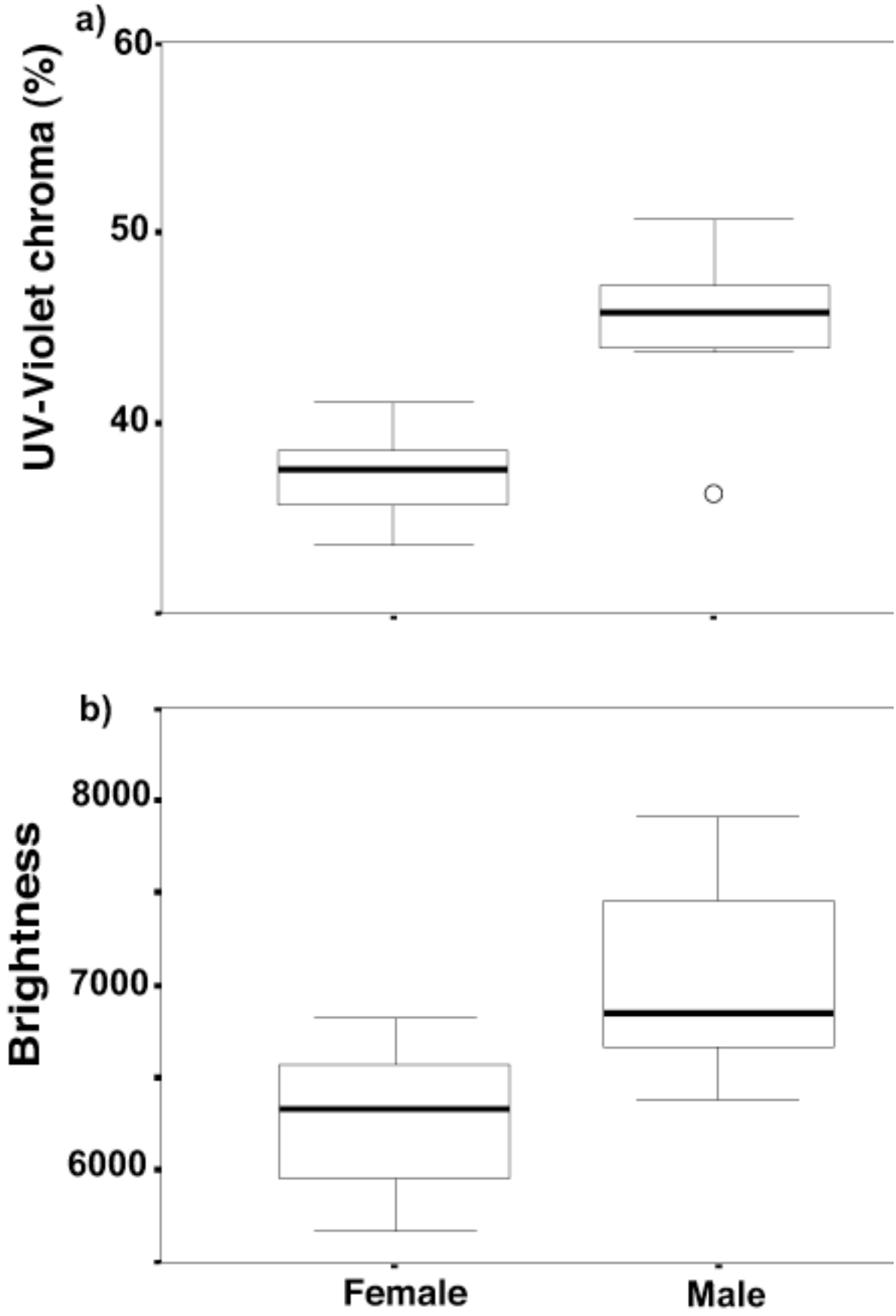
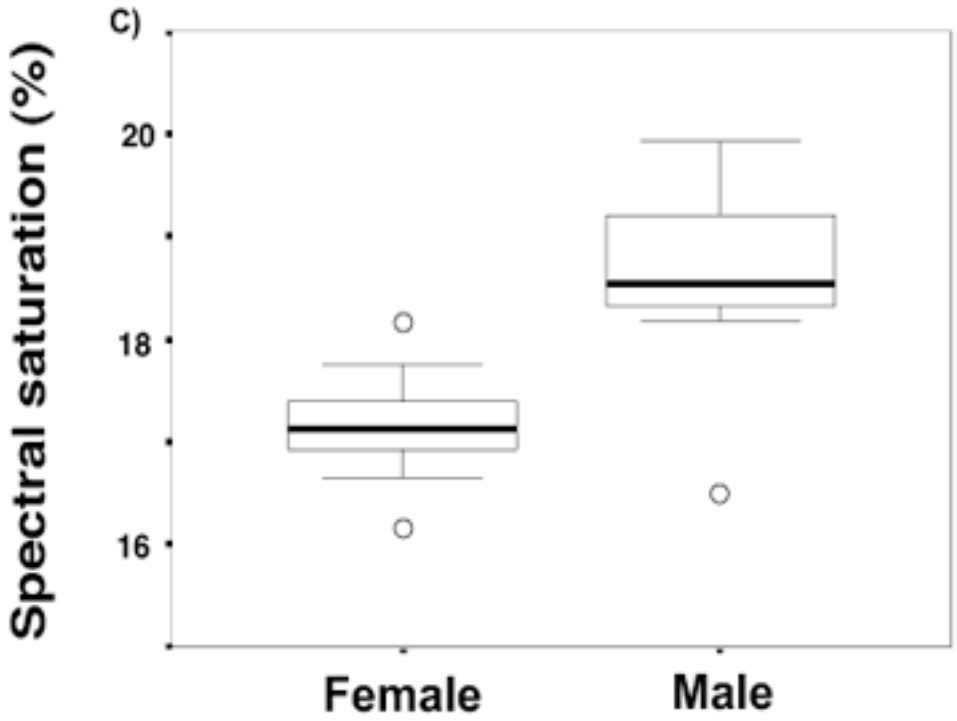
B
b)

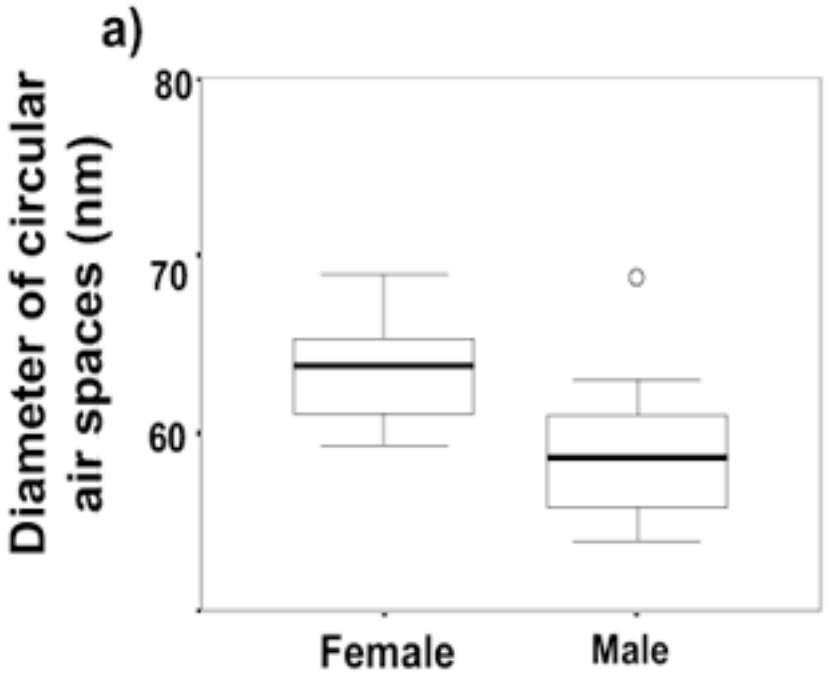
C)

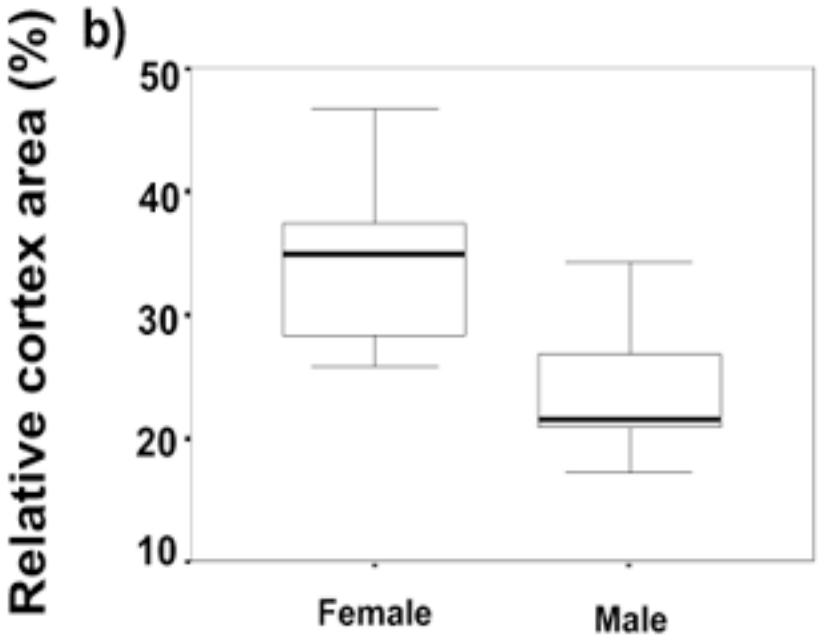


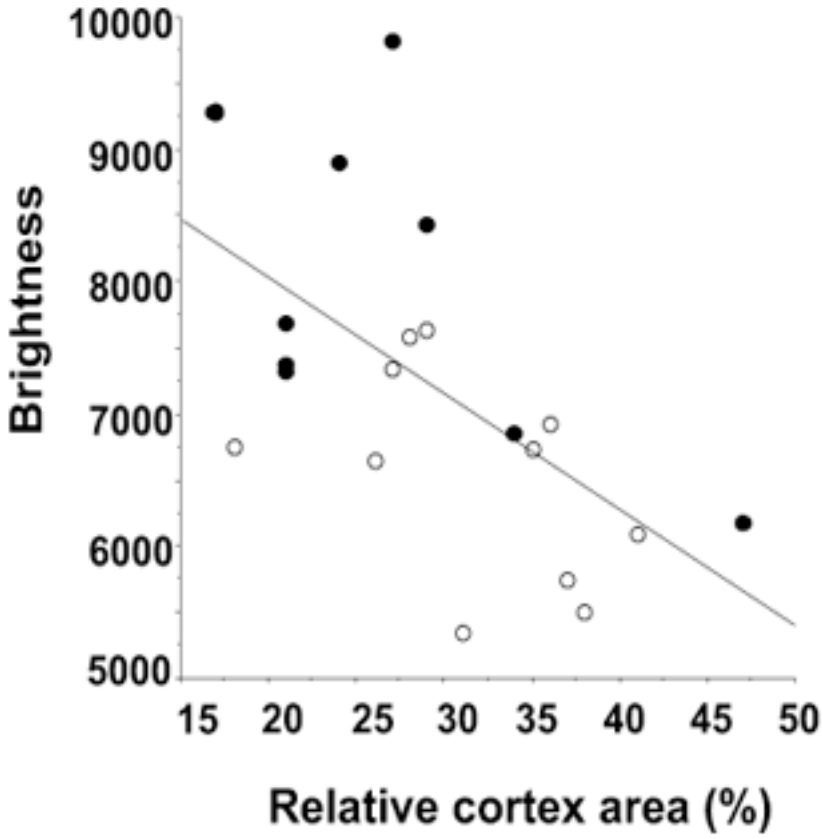

Log number of barbules
3.0
2.5
2.0
1.5
1.0
.5
0.0
Females
Males

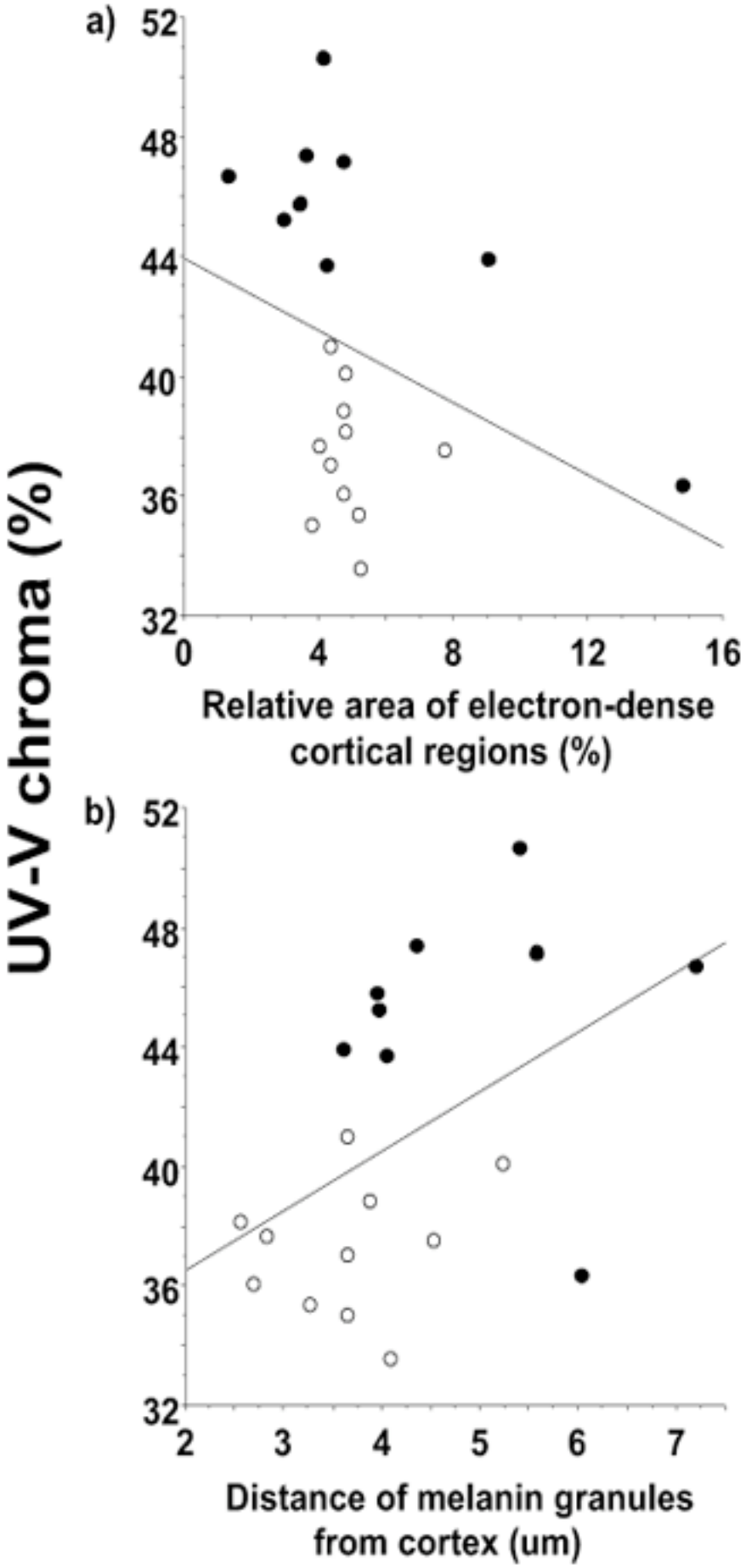

a)
UV-Violet chroma (%)
60
50
40
b)
Brightness
8000
7000
6000
Female
Male

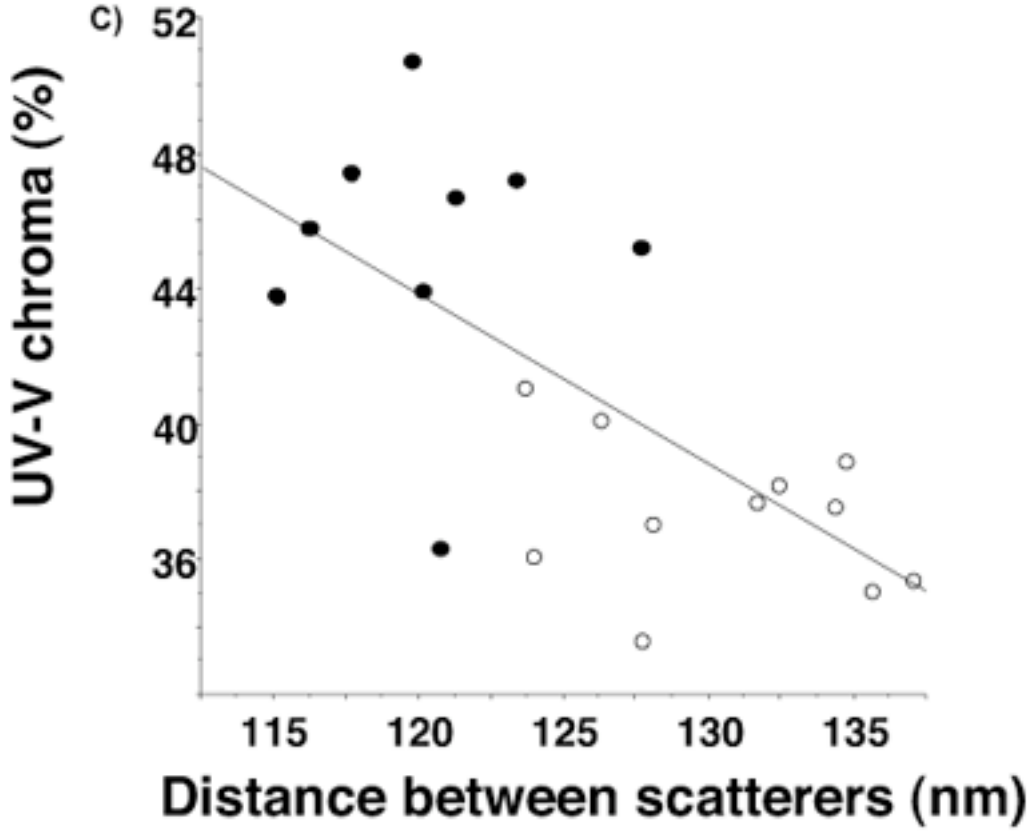

C)
Spectral saturation (%)
20
18
16
Female
Male

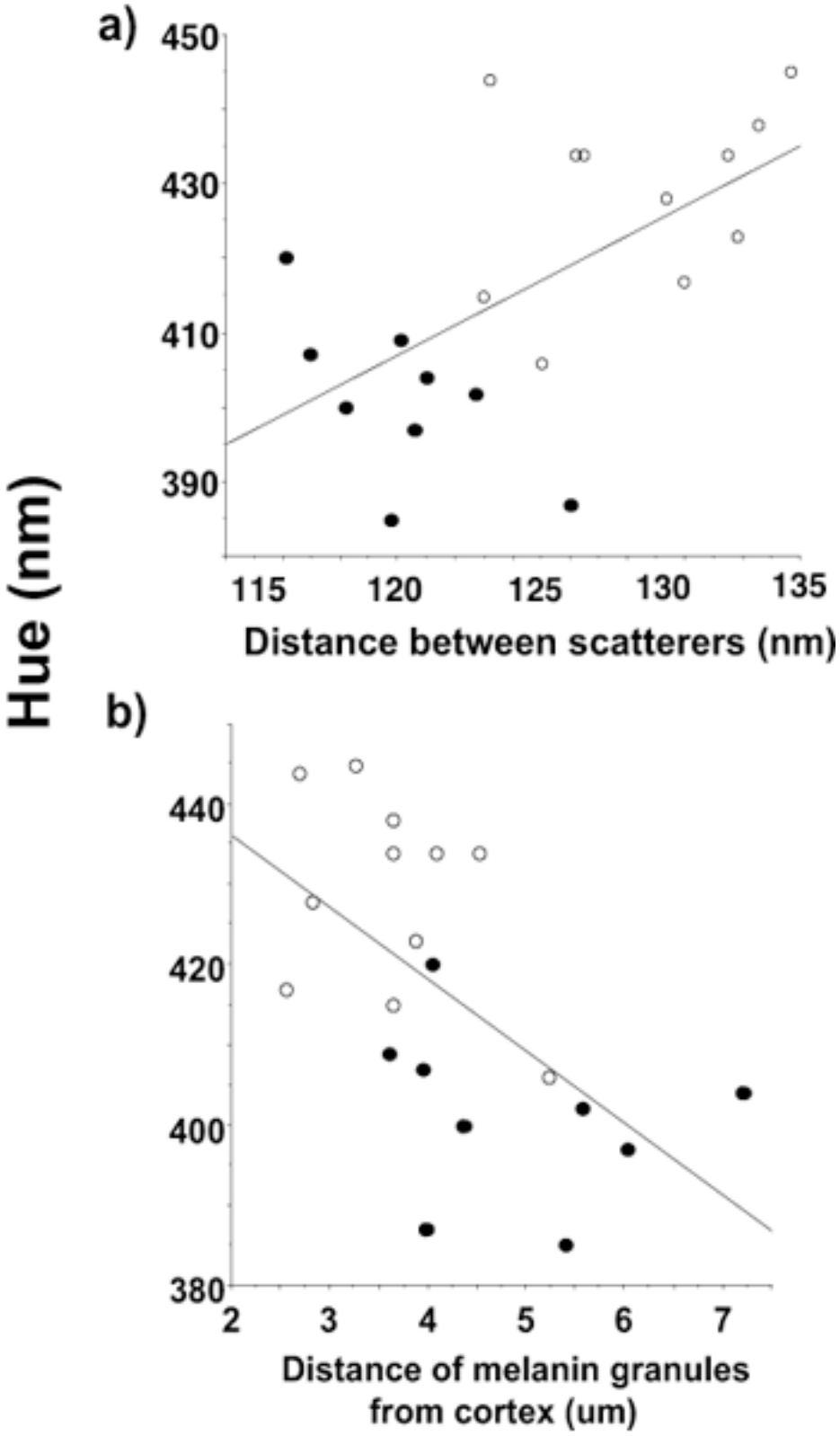

a)
Diameter of circular air spaces (nm)
80
70
60
Female
Male

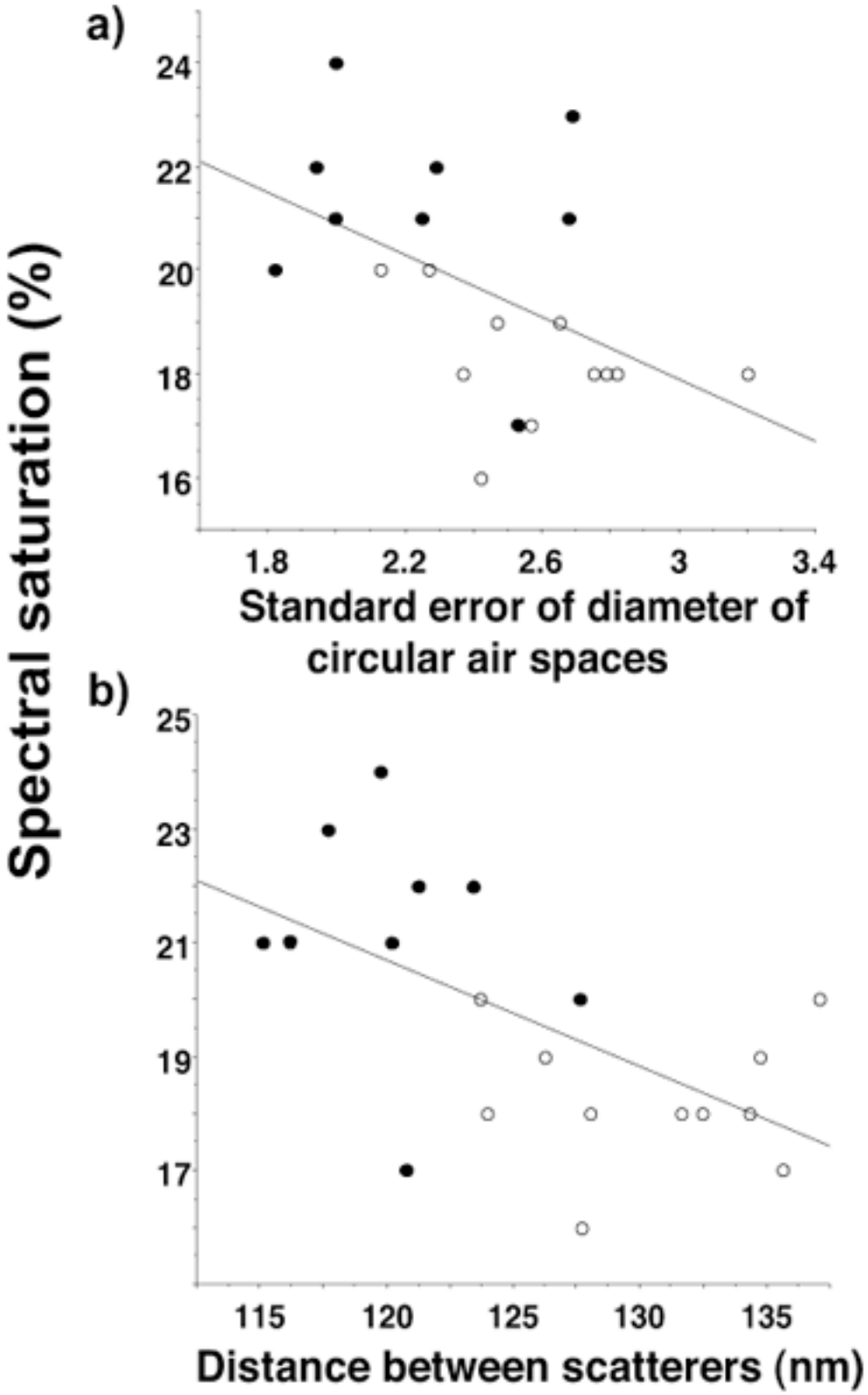

b)
Relative cortex area (%)
50
40
30
20
10
Female
Male


10000
9000
8000
7000
6000
5000
Brightness
15
20
25
30
35
40
45
50
Relative cortex area (%)


a)
52
48
44
40
36
32
0
4
8
12
16
Relative area of electron-dense cortical regions (%)
b)
52
48
44
40
36
32
2
3
4
5
6
7
Distance of melanin granules from cortex (um)
UV-V chroma (%)


c)
52
48
44
40
36
UV-V chroma (%)
115
120
125
130
135
Distance between scatterers (nm)


a)
450
430
410
390
115
120
125
130
135
Distance between scatterers (nm)
Hue (nm)
b)
440
420
400
380
2
3
4
5
6
7
Distance of melanin granules
from cortex (um)


a)
24
22
20
18
16
1.8
2.2
2.6
3
3.4
Standard error of diameter of circular air spaces
b)
25
23
21
19
17
115
120
125
130
135
Distance between scatterers (nm)
Spectral saturation (%)

Chapter 3

Significance of a basal melanin layer to production of non-iridescent structural plumage colour: evidence from an albino Steller's jay.

Abstract

1. Non-iridescent structural plumage color is typically produced by coherent scattering of light within a matrix of keratin and air ("spongy layer") in feather barbs.

2. It remains unclear what, if any, role the basal melanin layer underlying this spongy layer plays in the production of coloration.

3. Albinism in birds with structural color is a "natural experiment" in which a mutation causes the loss of melanin pigmentation, allowing us to identify the effects of the loss of melanin on structural color production.

4. Here we use full-spectrum spectrometry, transmission electron microscopy and Fourier analysis to compare the color and nanostructure of an albino Stellers' Jay (*Cyanocitta stelleri*) feather with a normal blue Steller's jay feather and, as a control, a white chicken (*Gallus gallus*) feather.

5. The albino jay feather had a washed out reflectance curve with a blue/green peak, while the blue feather had a typical bell-shaped blue curve with a UV/violet peak and the white feather had a typical white reflectance curve with no discrete peaks.

6. Electron microscopy revealed that both the albino and blue feather barbs contained well-formed spongy layers that were of the correct size and arrangement to produce their measured peak reflectance values, while the chicken feather had no spongy layer.

7. The washed out color of the albino jay feather was most likely caused by the loss of the basal melanin layer, suggesting that melanin functions to absorb incoherently scattered white light from the feather barb and thereby increase the purity of the color produced by the spongy layer.

Understanding the mechanisms by which color is produced in feathers can lead to insights into their signal content and evolution. 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; Hill and McGraw 2005). 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; Shawkey, Estes et al. 2003; Prum 2005). This medullary "spongy layer" lies beneath a keratin cortex and above a layer of melanin granules surrounding large central vacuoles. While 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 2005). 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 the Budgerigar (Simon 1971). Finally, melanin may serve no purpose in color

production and may only serve to provide rigidity to the feather (Burtt 1979) or some other function. None of these hypotheses has been tested.

Naturally occurring albino feathers provide a unique opportunity to test hypotheses for the function of the basal melanin layer in a bird that typically has structural coloration. Albino individuals lack melanin because of a disruption in the pathway of melanin synthesis that has no effect on other mechanisms in the body. Thus, by comparing albinistic to normal feathers, we can determine the effect of melanin removal on the color properties of these feathers.

In this paper, we use full-spectrum spectrometry and transmission electron microscopy (TEM) to compare the color and nanostructure of albino and normal blue Steller's Jay (*Cyanocitta stelleri*) feathers, and a normal white chicken (*Gallus gallus*) feather. First, we objectively measured the color of the three feathers using a spectrometer. If the absorbance hypothesis is correct, we predicted that the albino feather would reflect a washed out color, analogous to a television screen with a flashlight shining through it from behind (Prum 2005). If the backdrop hypothesis is correct, we predicted that the albino feather would reflect a light blue color (Simon 1971). If the null hypothesis is correct, we predicted that the albino feather would reflect the same color as the normal blue feather. Second, 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 the absorbance hypothesis is correct, we predicted that both the normal and albino feathers would have well-defined spongy layers with predicted hue values in the blue wavelengths. This would suggest that both the albino

and blue feathers should theoretically produce blue color, but that the lack of melanin granules in the albino feathers prevents the color from being fully expressed. If the backdrop or null hypothesis is correct, we predicted that the barbs of the albino feather would lack a spongy layer, and would resemble a barb from a normal white feather. The white color of the albino 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.*

Methods

A normally colored Steller's jay was observed molting into all-white plumage in the back yard of a resident in Boulder County, CO, USA (Schmoker, B., pers. comm.; see Figure 1). A tail feather from this bird were retrieved from an area in the backyard where the bird had been frequently observed and where no other white bird was ever seen; thus, we are certain of its origin. A blue tail feather was collected from a normally colored male Steller's Jay study skin. For further comparison, a white domestic chicken feather was collected from a pen at the Auburn University Poultry Science Department.

We used an Ocean Optics S2000 spectrometer (range 250-880 nm, Dunedin, FL, USA) to take reflectance measurements from these feathers. We placed feathers on gloss-free black construction paper, and chose analogous locations (~20 mm^2) on each for analysis. Using a block sheath that excluded ambient light, we held a bifurcated micron fiber optic probe at a 90° angle 5mm 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 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 arbitrarily chosen points within the selected locations on each feather.

From these reflectance curves we calculated several different color variables. We restricted these indices to wavelengths between 320 and 700 nm, as evidence suggests that passerine birds are sensitive to ultraviolet (UV) wavelengths (320-400 nm; (Cuthill, Partridge 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. (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 percentages of total light reflected in the ranges of 320-400, and 435-500 nm, respectively (Andersson, Ornborg et al. 1998), and are indices of color purity.

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

We scanned TEM micrograph negatives at 400 dpi using an Epson Perfection^{TM} 1240U flatbed scanner. We then analyzed the micrographs using Prum and 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, and at an appropriate scale, to produce color by coherent light

scattering alone (Prum, Torres et al. 1998; Prum, Torres et al. 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, we selected the largest available square portion of spongy layer (>500 pixels) uninterrupted by melanin granules, cell boundaries or keratin cortex. Because the barbs of white chicken feather lacked spongy layers, we selected the central vacuoles and the keratin surrounding them for analysis.

Because other microanatomical features of barb morphology other than the spongy layer may affect color production (Shawkey, Estes et al. 2005) we then used the program NIH Image v. 1.62 (available for download at http://rsb.info.nih.gov/nih-image) to measure additional structural components of the two colored barbs. We measured the thickness of the keratin cortex and spongy layer 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, the chicken feather was white with no overtones, the albino jay feather was white with a faint blue overtone and the normal jay feather was dark blue with black stripes. The reflectance spectra of the chicken feather was similar to that of the white feathers of other species of birds (Figure 2; for other examples of the reflectance spectra of white feathers see Mennill, Doucet 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 spectra of the albino feather was similar to that of the white feather (Figure 2). Unlike the white feather of a chicken or chickadee,

however, the albino feather had a single peak in the blue/green range followed by gradually decreasing reflectance. The reflectance spectra of the blue 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 discrete peak in the UV/violet range (Figure 2).

The hue of the albino 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 albino 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 structural colour (Figure 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 the peach-faced lovebird *Agapornis roseicollis,* Eastern bluebird *Sialia sialis* (Dyck 1971, Shawkey et al. 2003) and others. The albino feather differed from this blue feather in two ways (Figure3a,b). First, the basal layer of melanin granules was absent. Second, the keratin cortex of the albino feather was considerably thicker than that of the blue feather (Figure 3a,b; Table1). 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 (Figure 3c; Table 1).

Fourier analysis revealed that the spongy layers of the blue and albino feather barbs were sufficiently organized and at the correct scale to produce color by coherent light scattering alone. The discrete rings in the Fourier power spectrums (Figure 3d,e) indicate high levels of nanostructural organization (Prum et al. 1998, 1999). Fourier analysis of the spongy layer of these two feathers predicted hue values close to measured values (Figure 3g,h; Table 1). The predicted hue for the albino 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 (Shawkey et al. 2003; Prum et al. 1998, 1999, (Prum, Andersson 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 (Figure 3f). This lack of organization results in a lack of discrete peaks in the radial analysis (Figure 3i).

Discussion

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. While the role of melanin in non-iridescent avian structural color production has been the subject of speculation (Simon 1971; Prum 1999; Prum 2005), until now no test of function for this melanin layer has been conducted. Visual assessment, spectrometric, and nanostructural analyses all support the hypothesis that melanin primarily functions to absorb incoherently scattered white light from feather barbs. First, the albino feather was white with faint blue overtones, clearly contradicting the predictions of the null hypothesis. This simple visual assay suggests that loss of melanin has a clear and dramatic effect on

structural color production. 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 albino feather to be peaky and shifted to longer wavelengths than the blue feather. Instead, the reflectance spectra of the blue feather is distinctly peaky, while that of the albino curve is fairly saturated across all wavelengths and has only a suggestion of wavelength-specific reflection in the blue/green wavelengths. The higher UV chroma value of the blue feather further suggests that it reflects a more pure, saturated color. These results appear to confirm the predictions of the absorbance hypothesis and not the predictions of the backdrop hypothesis. However, additional changes in the microstructure of the albino feather barbs, such as loss of spongy layer, may explain its washed-out appearance.

Our microstructural analyses, however, indicate that the albino feather has a well-defined spongy layer that is organized at the proper scale to produce a blue/green color. The fact that this albino 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 green 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 albino 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; Shawkey et al. 2005). The thicker cortex of the albino 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. While 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, Brandt 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 (Brink and van der Berg 2004) showed 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. More theoretical and empirical work on the mechanisms that create structural color display is needed. In particular, 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 tinkered with over evolutionary time to create the amazing diversity of structural color found in birds.

Acknowledgements

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Ornithologists' Union to M.D.S, and National Science Foundation grants DEB007804 and IBN0235778 to G.E.H.

Figure legends

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

Figure 2: Raw (a) and normalized (b) reflectance spectra of an albino 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 (a) overall reflectance and (b) the shapes of the curves between feathers.

Figure 3: Feather microstructure, Fourier power spectra and predicted reflectance spectra of a blue Steller's jay feather (a,d,g), an albino Steller's jay feather (b,e,h) and a white chicken feather (c, f, i). Shown are TEM micrographs of barbs (x1900, a,b,c, Scale bar = 1 μm) with insets showing close-ups of spongy layer (x9100, a,b; scale bar = 500 nm), Fourier power spectra (c,d,e) and predicted reflectance spectra based on Fourier analysis. C = cortex, SL = spongy layer, M = melanin granules, V = vacuoles.

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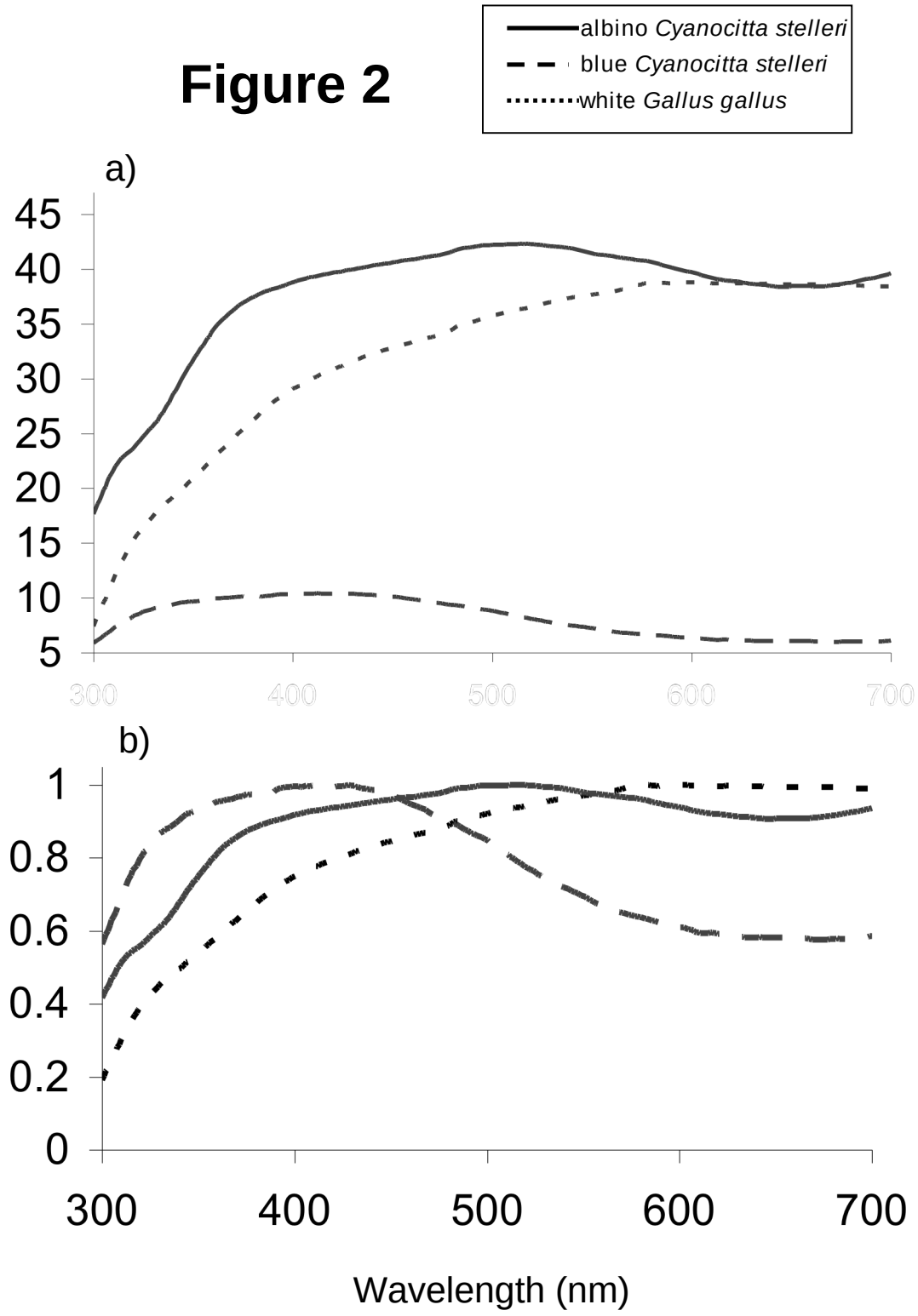
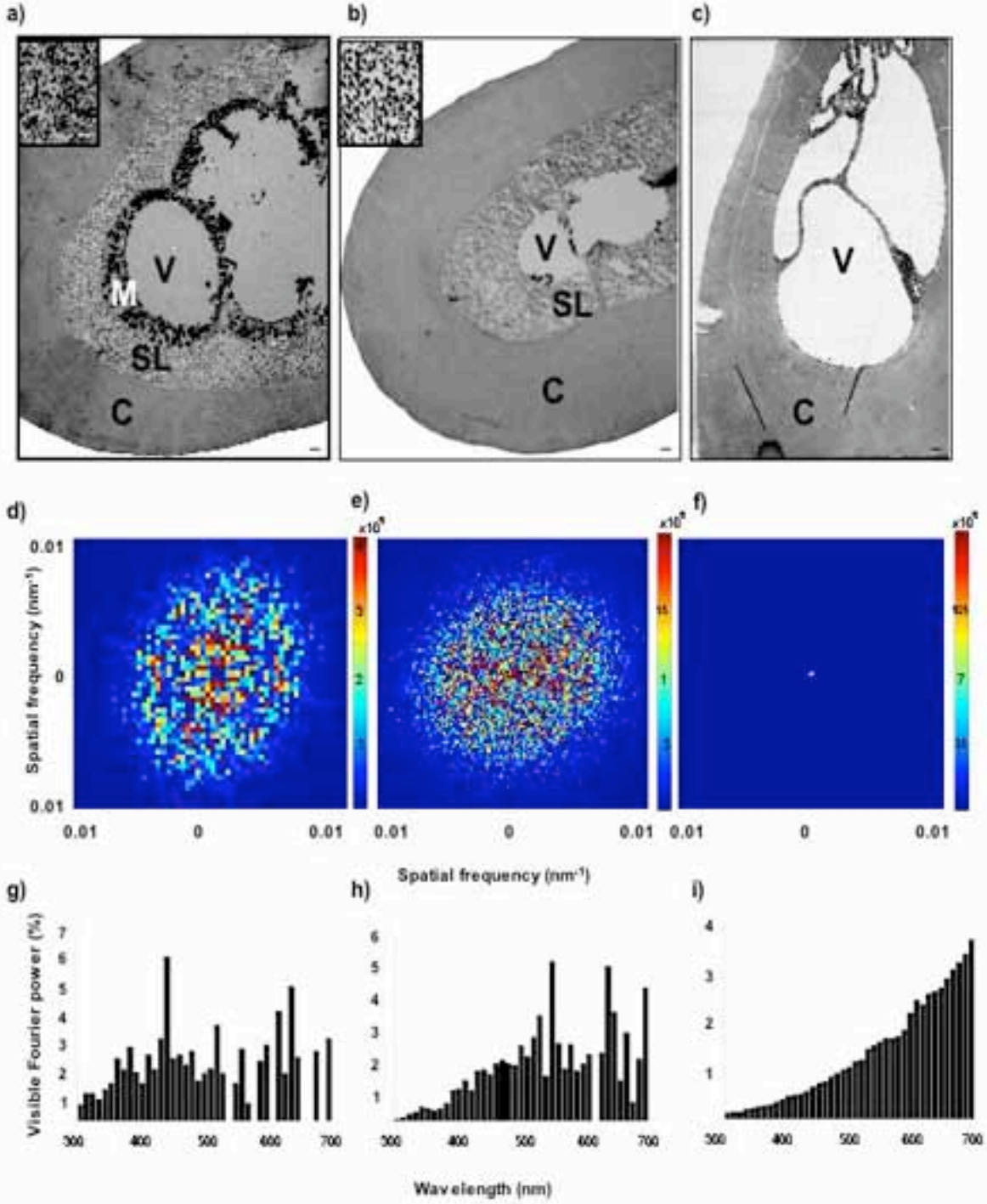
Table 1: Color and morphologocial measurements of an albino Steller's jay feather, a normal blue Steller's jay feather and a white chicken feather.

	Hue (nm)	Predicted hue (nm)	Brightness (%)	UV-V Chroma (%)	Blue Chroma (%)	Spectral saturation (%)	Spongy layer thickness (nm)	Cortex thickness (nm)
Blue feather	413	441	8.08	27.20	33.28	30.72	4474.41	8383.77
Albino feather	514	493	37.93	20.37	30.37	26.44	6313.93	12052.33
White feather	n/a	n/a	32.94	15.92	28.81	28.19	n/a	7386.47

Figure 1

Figure 2


albino *Cyanocitta stelleri*
blue *Cyanocitta stelleri*
white *Gallus gallus*
a)
45
40
35
30
25
20
15
10
5
300
400
500
600
700
b)
1
0.8
0.6
0.4
0.2
0
300
400
500
600
700
Wavelength (nm)


a)
b)
c)
V
M
SL
C
V
SL
C
V
C
d)
e)
f)
Spatial frequency (nm⁻¹)
0.01
0
0.01
Spatial frequency (nm⁻¹)
g)
h)
i)
Visible Fourier power (%)
Wavelength (nm)
300
400
500
600
700

Chapter 4

Microbial diversity of wild bird feathers revealed through culture-based and culture-independent techniques.

Abstract

Despite recent interest in the interactions between birds and environmental microbes, the identities of the bacteria that inhabit the feathers of wild birds remain largely unknown. We used culture-based and culture-independent surveys of the feathers of eastern bluebirds (*Sialis sialis*) to examine bacterial flora. When used to analyze feathers taken from the same birds, the two survey techniques produced different results. Species of the poorly defined genus *Pseudomonas* were most common in the molecular survey, while species of the genus *Bacillus* were predominant in the culture-based survey. This difference may have been caused by biases in both the culture and PCR techniques that we used. The pooled results from both techniques indicate that the overall community is diverse and composed largely of members of the *Firmicutes* and β- and γ- subdivisions of the *Proteobacteria*. For the most part, bacterial sequences isolated from birds were closely related to sequences of soil- and water-borne bacteria in the Genbank database, suggesting that birds may have acquired many of these bacteria from the environment. However, the metabolic properties and optimal growth requirements of several isolates suggest that some may have a specialized association with feathers.

Introduction

The identities and ecological roles of microbes found on the feathers of wild birds are largely unknown. For a variety of reasons, we might expect to find a limited diversity and abundance of bacteria on the surface of feathers. First, birds waterproof their feathers by applying preen oil (12), thereby limiting water availability. This oil inhibits the growth of some bacteria, although it appears to enhance the growth of others (3, 27). Second, common proteolytic enzymes produced by bacteria can not degrade the ß-keratin sheets that constitute 90% of feather mass (37). Thus, to utilize feathers as a nutrient source, bacteria must produce keratinolytic enzymes that convert feather keratin to peptides (37). Such enzymes appear to be produced by fairly diverse groups of bacteria in the environment (15), but whether these bacteria are also present and active on wild bird feather is unknown. Bacteria may also use detritus or other microbes on feathers as nutrient sources, but such use has yet to be documented. There have been no inventories of the microbiota of the feathers of a wild bird, and the ecology of microbes on feathers cannot be understood until such basic inventories are obtained.

Burtt and Ichida (6) isolated keratinolytic *Bacillus* spp. from a broad spectrum of birds. Shawkey *et al.* (27) cultured thirteen distinct isolates (determined by BLAST searches of 16S rDNA sequences) from the feathers of house finches (*Carpodacus mexicanus*). To our knowledge, however, no one has yet comprehensively characterized the microbial communities living on feathers of any species. Such a survey is needed for several reasons. First, these basic data are needed to determine how microbes interact both with one another and with birds. For example, Burtt and Ichida (6) suggested that

degradation of feathers by *Bacillus* sp. may have partially driven the evolution of feather molt. They used highly selective media, and therefore did not isolate any non-*Bacillus* strains. However, different species of bacteria may act syntrophically to degrade feathers, or, conversely, the metabolic activity or antibiotic production of some bacteria may inhibit the growth of others. Some bacteria on feathers could be parasites, as suggested by Burtt and Ichida (6) while others could be commensals or even mutualists. Such communities are seen on the human skin, where the activity of bacteria utilizing the sebum of the skin lowers the pH of the skin's surface, providing an effective barrier preventing colonization of other (possibly pathogenic) bacteria (35). Second, all studies of feather bacteria to date have relied on culture-based methods. A very small proportion (<1%) of microbes can be cultured by traditional methods (2), so culture-based studies may not provide an adequate sampling of diversity. The use of culture-independent molecular phylogenetic techniques allows us to sample a broader spectrum of bacteria on feathers, although these methods have several limitations (8, 22, 31, 32, 38) including the possibility that DNA isolation, amplification, and cloning might be biased in favor of certain phylogenetic groups. By simultaneously performing molecular and culture-based sampling, we can compare results obtained using the two methodologies.

In the current study, we characterized the bacteria on the feathers of a common North American passerine, the eastern bluebird (*Sialia sialis*), using both culture-based and culture-free methods. The purpose of this study was to describe the diversity of bacteria found on feathers and to compare data collected using culture-based and culture-free methods.

Methods

Collection of materials

In July 2003, we trapped two adult male (M1 and M2) and two adult female (F1 and F2) Eastern Bluebirds (*Sialia sialis*) in Lee County, Alabama (32°35'N, 82°28'W) using mist nets and box traps. Wearing sterile rubber gloves, we pulled contour feathers from the breast, belly and rump of the birds and placed them in separate sterile tubes. All tubes were transported to the laboratory within 3 hr and processed immediately. For each bird, we created two sets of 15 feathers using 5 feathers from each of the 3 body areas sampled. One set was analyzed using culture-independent methods and the other was analyzed by the culture-based method.

Culture-independent method

DNA extraction, amplification, and cloning

The feathers from each bird were homogenized in liquid nitrogen using a sterile mortar and pestle (39), and resuspended in 10 ml sterile 0.85% NaCl solution. After settling of particulate matter, 1 ml of the suspension was transferred to a sterile microcentrifuge tube and pelleted for 1 minute at 13,000 x *g*. The supernatant was removed and the pellet was re-suspended in 100 μl of sterile 0.85% NaCl. DNA was extracted from the pellet using the DNeasy® Tissue Kit (Qiagen, Valencia, CA), and used as a template for PCR amplification of the bacterial 16S rDNA gene.

To generate libraries of PCR-amplified 16S rDNA sequences from DNA isolated from feathers, we used "universal" primers 515F (5'- GTGCCAGCMGCCGCGGTAA-3') and 1492R (5'- GGTTACCTTGTTACGACTT- 3') (5). All PCRs were performed in 50 μl reaction volumes containing between 1 and 50 ng purified DNA template and (as final concentrations) 1x PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTP's, 100 μM of each

forward and reverse primer, and 0.025 U/μl Ampli*Taq* DNA polymerase (Applied Biosystems, Foster City, CA). All reactions were incubated on a model PT-100 thermal cycler (MJ Research, Inc., Waterstown, MA) for an initial denaturation step at 94° C for five minutes, followed by 36 cycles of denaturation (94° C, 1 min), annealing (55° C, 1 min) and extension (72° C, 2 min). An extension step of 10 min at 72° C was added after the last cycle to promote A- tailing of PCR products prior to cloning. Three reactions were performed for each sample. PCR products were checked on 1.5% agarose gels and the 1-kbp amplicons were excised from gels and purified with the Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. 20-50 ng of this purified PCR product was cloned into the pCR-II® TOPO® vector (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

Plasmid DNAs containing inserts were amplified by colony PCR using either the vector primers M13F and M13R, or primers M13F and 1492R in 50 μl reaction volumes containing 1X PCR buffer, 2.5 mM $MgCl_2$, 400 μM dNTP's, 400 μM each forward and reverse primer, and 0.025 U/μl Ampli*Taq* polymerase (Applied Biosystems, Foster City, CA). Recombinant colonies were inoculated directly in the PCR mixture using sterile pipette tips. The PCR consisted of a denaturing step (94° C, 5 min), followed by 36 cycles of denaturation (94° C, 1 min), annealing (52° C, 1 min) and extension (72° C, 2 min), and a final extension step (72° C, 5 min). To verify the success of PCR, 7 μl of each PCR product was checked by electrophoresis.

RFLP screening of rDNA clones

To avoid sequencing redundant clones, we screened clones using an restriction fragment length polymorphism (RFLP) analysis. Aliquots (10 μl) of crude PCR product

were digested to completion with the restriction enzymes *Msp*I and *HinP*1 I in 1X NEB buffer 2 (New England Biolabs, Beverly, MA) in a final volume of 20 μl for three hours at 37° C. Digested products were separated on agarose gels (4% MetaPhor®, Cambrex, Baltimore, MD). Using digital images of these gels in Adobe Photoshop® 4.0 LE, we aligned the RFLP patterns obtained for each bird visually, and selected representatives from each group for sequencing.

The PCR products from representative clones were sequenced at the Auburn University Genomics and Sequencing Laboratory using the M13F and 1492R primers.

Phylogenetic analyses

Sequences were inspected manually for the presence of ambiguous base assignments, and chimeric sequences were identified using the Chimera Check program in the Ribosomal Database Project (RDP; 16). The BLAST algorithm (1) was used to determine their approximate phylogenetic affiliation. Sequences were then aligned with known rDNA sequences in the RDP using the Sequence Match function. All chimeras and sequences with >99% similarity to known PCR contaminants (34) were discarded. Sequences that were ≥ 99% similar to one another were considered as a single relatedness group, and we chose the most complete and unambiguous representative for further analysis. Unique bacterial 16S rDNA sequences were deposited in GenBank (accession numbers AY581128-AY581144).

Unique sequences were then compiled with known sequences of ATCC type cultures from GenBank and the RDP in MacClade v. 4.0 (17), and aligned in ClustalX v.1.83 (36). Only homologous nucelotide positions with unambiguous bases in every

sequence were used in phylogenetic analyses. Distance-based methods were used to construct bootstrap neighbor-joining trees in PAUP* 4.0b10 (33). Separate trees were constructed for each major phylogenetic subdivision (Firmicutes, α-Proteobacteria, γ-Proteobacteria).

Culture-based method

The second set of feathers from each bird was homogenized in sterile phosphate-buffered saline using a sterile all-glass tissue grinder (Kontes, Vineland, NJ). 100 μl of raw homogenate, as well as serial dilutions, was transferred onto two distinct media. Tryptic soy agar (TSA, Difco, New Jersey) is a generalized medium while feather meal agar (FMA; 15 g l^{-1} feather meal, 0.5 g l^{-1} NaCl, 0.30 g l^{-1} K_2HPO_4, 0.40 g l^{-1} KH_2PO_4, and 15 g l^{-1} agar), is selective for keratinolytic bacteria (25). Both media types contained 100 μg/ ml of cycloheximide to inhibit fungal growth (29), and all plates were incubated at room temperature (~28° C) for one week. The rationale for selecting this growth temperature was that mesophilic bacteria should all grow at a temperature of 28° C.

Eighty colonies were chosen at random. Colonies with unusual or infrequently detected morphologies were always selected, to increase the probability of obtaining a diverse sampling. Colonies were re-streaked on TSA at least three times, and incubated at room temperature for 48-72 hours each time until the purity of culture was confirmed by examination of colony morphology.

Pure cultures were re-streaked on TSA and incubated at 28° C for 48 hours in preparation for identification. A loopful of cell material of late-log phase cells was harvested, and fatty acids were extracted and methylated according to the procedure described by the manufacturer (Microbial ID, Inc., Newark, DE). Samples were analyzed

using a Hewlett-Packard (Palo Alto, CA) 5890 series II gas chromatograph with a 7673 autosampler, a 3396 series II integrator, and a 7673 controller. Using the Sherlock (Microbial ID, Inc.) program on a Hewlett-Packard Vectra QS/20 computer, the chromatograms were compared to a database of reference cultures previously grown on TSA.

Results

Molecular phylogenetic method

Using RFLP, approximately 220 clones were screened from each library for a total of 909 clones. Typically, 5 to 15 bands resulted from each rDNA digest in the discernible fragment size range of 50 to 300 bp (Figure 1). Twenty unique banding patterns in library F1, 25 in F2, 34 in M1, and 18 in F2 were detected. When a banding pattern was faint or unclear, the corresponding PCR product was sequenced.

Figure 1 goes here

The chimera-detection program of the RDP was used to detect chimeras. The most serious limitation of this program is that it depends on the presence of sequences of the parent molecules in the database. Because the sequences in this study were for the most part closely related to known sequences (see below), this limitation should not affect the detection of chimeras in our samples. Two chimeric sequences were detected and discarded, and one sequence was identified as a known contaminant (34) and discarded. The BLAST program identified 14 different 16S rDNA gene sequences as the closest relatives of the feather bacteria sequences (Table 1). Approximately 53% of the

unique sequences obtained were between 98 and 100% identical to their closest matches in GenBank. Approximately 35% of the sequences obtained were between 95 and 97% identical to their nearest match, and 12% were ≤93% identical. Seventeen unique sequences were used in subsequent phylogenetic analyses.

Although all sequences identified were representatives of the eubacteria, the overall community was diverse. The majority (10/17 or 62%) of unique sequences used in phylogenetic analyses were representatives of the γ subdivision of the Proteobacteria (Table 1; Figure 2). Many of these sequences were most closely related to bacteria of the genus *Pseudomonas*, but this genus is poorly defined, with representatives in the α, β, and γ subdivisions of the Proteobacteria (14). Most of our *Pseudomonas*-like sequences were closest to the fluorescent Pseudomonads in the γ-Proteobacteria (Figure 2a). Approximately 23% (4/17) of unique sequences were closely related to bacteria in the β subdivision of the Proteobacteria (Figure 2b). The remaining unique sequences (3/17 or 16%) were most closely related to bacteria in the Firmicute division (Figure 2c).

Figure 2 goes here

Culture-based method

Eighty randomly picked colonies were analyzed using gas chromatography of cellular fatty acids. Similarity indices ranged from 0.140 to 0.904, with an average of 0.491, which is considered robust for this type of analysis (30). Identifications with similarity values <0.300 (11 samples) were not used in any analysis. Firmicutes represented the largest portion (50 of 69 or 72%, table 2) of the identified organisms,

followed by γ-Proteobacteria (17 of 69 or 25%, table 2), α-Proteobacteria and Actinobacteridae (both 1/69 or 1.4%).

Table 2 goes here

These results stand in sharp contrast to those obtained using the culture-independent method. The difference between the results obtained by these methods was unexpected, so the possibility that either a) the GC results were inaccurate or b) we were unable to amplify the sequences corresponding to the GC results was tested. Ten colonies that had been analyzed by gas chromatography were grown overnight on TSA, and 16S rDNA sequences were obtained from them using PCR with primers 515F and 1492R. These sequences were compared to known sequences in GenBank using BLAST and the results are displayed in Table 2. Most of the 16S rDNA sequence identifications corresponded to the GC identifications, indicating that we were able to amplify sequences from these organisms and that our GC results were accurate.

Discussion

The primary purpose of this study was to inventory the microbial diversity of wild bird feathers. In other studies researchers have focused on particular groups of microbes (e.g. *Bacillus*, 6) or on keratinolytic bacteria (27), but here we surveyed total bacterial diversity using both culture-based and culture-independent methods. Such a survey is a necessary first step towards a fuller understanding of the microbial ecology of bird feathers and the potential interactions of birds and feather microbes. Members of the genus *Pseudomonas* were most heavily represented in the molecular analysis, while

members of the genus *Bacillus* were most heavily represented in the culture-based analysis. Given the simple nutritional requirements and growth conditions of the *Pseudomonas* spp. from our molecular survey, it is surprising that we did not detect them in our culture-based survey. Perhaps the number of isolates we identified was too small for detection. However, the apparent numerical dominance of *Pseudomonas*-like sequences in our molecular survey suggests that they should also be widespread in our culture-based survey. Similarly, we would expect the dominance of *Bacillus* in our culture-based method to be reflected in the results of our molecular survey. These conflicting results may be caused by the biases of both PCR- and culture-based methods. The guanine-plus-cytosine (G+C) content of template DNA has been reported to influence gene amplification by PCR (7, 23). Reysenbach *et al.* (23) found that low G+C rDNA was preferentially amplified from a mixture of low and high G+C rDNA. Firmicutes of the genus *Bacillus* have fairly low G+C content (~40-48%), while many of the *Pseudomonas* spp. that we identified, in particular *P. fluorescens*, have high G+C content (~66% for *P. fluorescens*). Thus, our pattern is the opposite of what we expected from G+C content bias. Our successful amplification of DNA isolated from cultured *Bacillus* sp. using the Qiagen kit suggests that the bias was not introduced at the DNA-extraction phase. Differences in secondary structure affecting either the availability of the priming sites or the polymerization reaction may cause amplification bias (11). In any case, our results emphasize that either multiple primer pairs or both molecular and culture-based approaches should be used when characterizing microbial communities (11, 26).

Taken together, our methods identified a diverse group of bacteria from the feathers of eastern bluebirds. Many of the organisms were closely related to common soil bacteria. *P. fluorescens* is a highly heterogenous "species" that can be subdivided by various taxonomic criteria into subspecies, biotypes or biovars (20). Indeed, *P. lundensis* was independently discovered as both a separate species (18) and as a well-defined subgroup of *P. fluorescens* (4). The various forms of *P. fluorescens*, along with the other related *Pseudomonas* species in this study appear to be ubiquitous in soil (20). *Janthinobacterium* spp. are also commonly found in soil and water (13, 21), although they typically comprise only a small portion of the total microflora. This distribution suggests that birds may acquire them while landing or foraging on the ground.

The metabolic properties and optimal growth requirements of some of the identified bacteria suggest that they may play roles in the ecology of feathers. Members of the genus *Lactobacillus* are extremely fastidious organisms (10), so it is not surprising that we did not obtain them in culture. The optimal growth of many *Lactobacillus* spp. under microaerobic conditions suggests that they may reside on the portion of the rachis lying beneath the skin. Similarly, aerotolerant anaerobes of the genus *Streptococcus* may reside near the skin, as they do in humans (24). Further analyses of different sections of feathers are needed to investigate the location of these bacteria.

One of our sequences was closely related to *Rhodoferax ferrireducens*, a recently isolated bacterium capable of breaking down acetate (9), most likely using the glyoxylate bypass pathway. This pathway may enable the bacteria to break down the components of preen oil on feathers. If so, this raises the possibility that preen oil may inhibit the

growth of some bacteria while providing a carbon source for others. Other studies have provided evidence of this type of effect *in vitro* (3, 27).

Most studies of feather bacteria have focused on the genus *Bacillus* (6, 19), because strains of *B. licheniformis*, *B. pumilus*, and *B. megaterium* have been shown to have significant keratinolytic activity *in vitro*. *Bacillus* spp. were fairly abundant in our culture-based survey (see Table 2), suggesting that they may be common inhabitants of bird feathers. Strains of *Kocuria kristinae* have similar keratinolytic properties (27). Further tests are needed to determine whether these bacteria can colonize feathers and express keratinolytic enzymes on feathers of live birds. Because they can form spores, *Bacillus* spp. may reside on feathers in a resting state, and may not become active until feathers are molted and drop to the ground. Like *Pseudomonas*, these species are common in soil (28), and this may be the source of acquisition by birds.

Our results show that the microbial composition of bird feathers is diverse. The interactions of these bacteria with one another and, potentially, with birds themselves should prove a fascinating avenue for continued research. First, we need to determine which bacteria are active on feathers and how they acquire nutrition, whether from the feathers themselves, from detritus, from other microbes, or from other sources yet to be identified. Second, we should investigate interactions between bacteria on the feathers of birds and determine how bacterial communities may be controlled through, for example, the application of preen oil. Finally, we should examine whether these communities can affect birds through the degradation of feathers or possibly by acting as opportunistic pathogens. By doing so, we may gain some insight into the ecological roles of these bacteria and their potential co-evolution with birds.

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Table 1: Identification of unique cloned bacterial sequences from the feathers of eastern bluebirds in Lee County, AL. The species in GenBank with the closest DNA sequence to each isolate (as determined by the BLAST algorithm) is presented as a preliminary identification.

Clone	Highest BLAST identity (Accession number)	Base pairs matched	Division	RF pat det fre
9	*Pseudomonas poae* (AJ492829)	752/765 (98%)	γ-proteobacteria	36
432	*Stenotrophomonas maltophila* (AF137357)	927/937 (99%)	γ-proteobacteria	45
753	*Acinetobacter venetianus* (AVE295007)	763/790 (96%)	γ-proteobacteria	34
73	*Pseudomonas brennerii* (AF268968)	771/784 (98%)	γ-proteobacteria	9
791	*Ewingella americana* (U29438)	950/967 (98%)	γ-proteobacteria	33
85	*Pseudomonas lundensis* (AB021395)	941/961 (98%)	γ-proteobacteria	47
78	*Aeromonas veronii* (X60414)	788/802 (98%)	γ-proteobacteria	10
15	*Pseudomonas fluorescens* (AF094725)	951/959 (99%)	γ-proteobacteria	305
531	*Pseudomonas fragi* (AF094733)	806/840 (95%)	γ-proteobacteria	85
2	*Pseudomonas lundensis* (AB021395)	871/897 (97%)	γ-proteobacteria	67
60	*Janthinobacterium lividum* (AF174648)	836/837 (96%)	β-proteobacteria	53
252	*Rhodoferax ferrireducens* (AF435948)	706/752 (93%)	β-proteobacteria	20
47	*Janthinobacterium lividum* (AF174648)	766/802 (95%)	β-proteobacteria	35
96	*Janthinobacterium lividum* (AF174648)	699/710 (98%)	β-proteobacteria	20
88	*Streptococcus vestibularis* (AY188353)	765/772 (99%)	Firmicutes	5
4	*Streptococcus salivarius* (AF459433)	785/807 (97%)	Firmicutes	7
191	*Lactobacillus gasseri* (AF519171)	806/863 (93%)	Firmicutes	10

Table 2: Cultured isolates from the feathers of four eastern bluebirds (*Sialia sialis*) captured in Lee County, Alabama. Isolates were identified by gas chromatography of cellular fatty acids and, in some cases, by BLAST searches of 16S rDNA sequences.

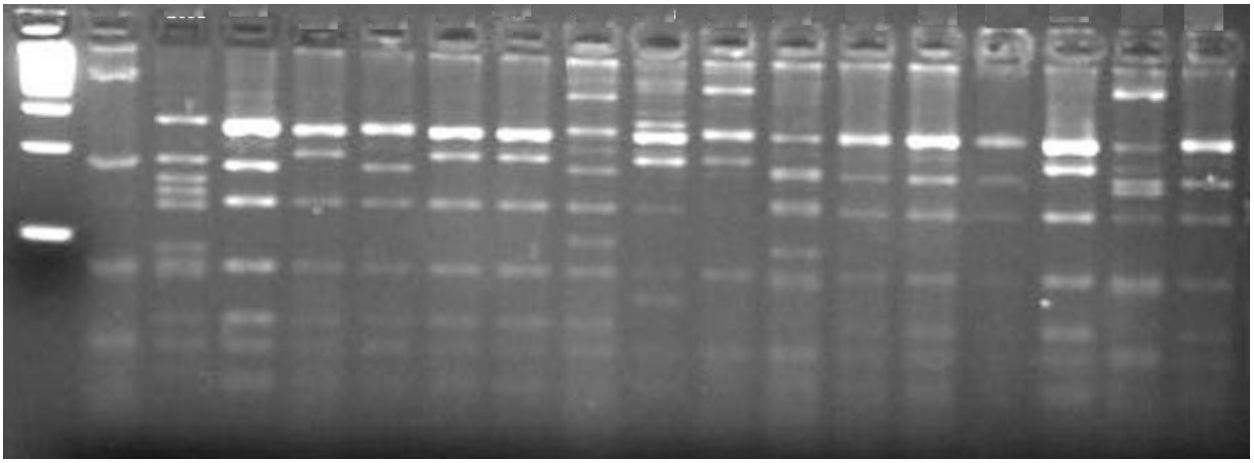
Identification	Division	Number of isolates	Highest BLAST identity (Accession number)
Acinetobacter calcoaceticus	Firmicutes	1	*Acinetobacter calcoaceticus* (M
Arthrobacter mysorens	Firmicutes	1	
Bacillus cereus	Firmicutes	14	*Bacillus anthracis* (AY043083)
Bacillus licheniformis	Firmicutes	4	*Bacillus cereus* (Z84581)
Bacillus mycoides	Firmicutes	6	
Bacillus pumilus	Firmicutes	15	*Bacillus sp.*(X81132)
Bacillus sphaericus	Firmicutes	1	
Cellulomonas flavigena	Firmicutes	1	
Exiguobacterium acetylicum	Firmicutes	6	*Exiguobacterium oxidotolerum* (AB105164)
Enterococcus durans	Firmicutes	1	*Bacillus cereus* (Z84581)
Microbacterium liquefaciens	Actinobacteridae	1	*Microbacterium esteraromaticu* (AB099658)
Erwinia chrysanthemi	γ-proteobacteria	1	
Pantoea ananas	γ-proteobacteria	1	
Serratia marcescens	γ-proteobacteria	15	*Serratia marcescens* (AY39501
Sphingomonas capsulata	α-proteobacteria	1	

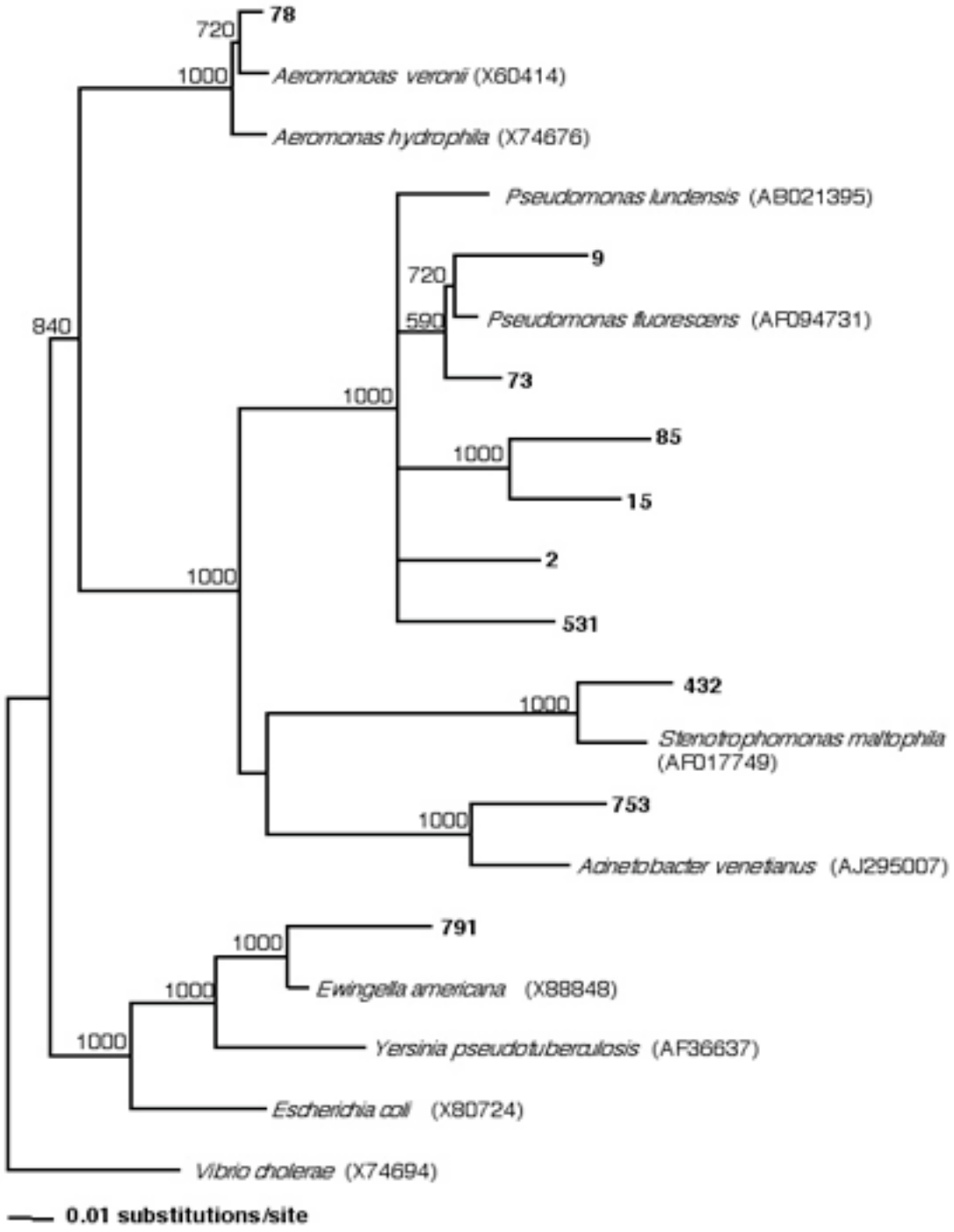
Figure legends

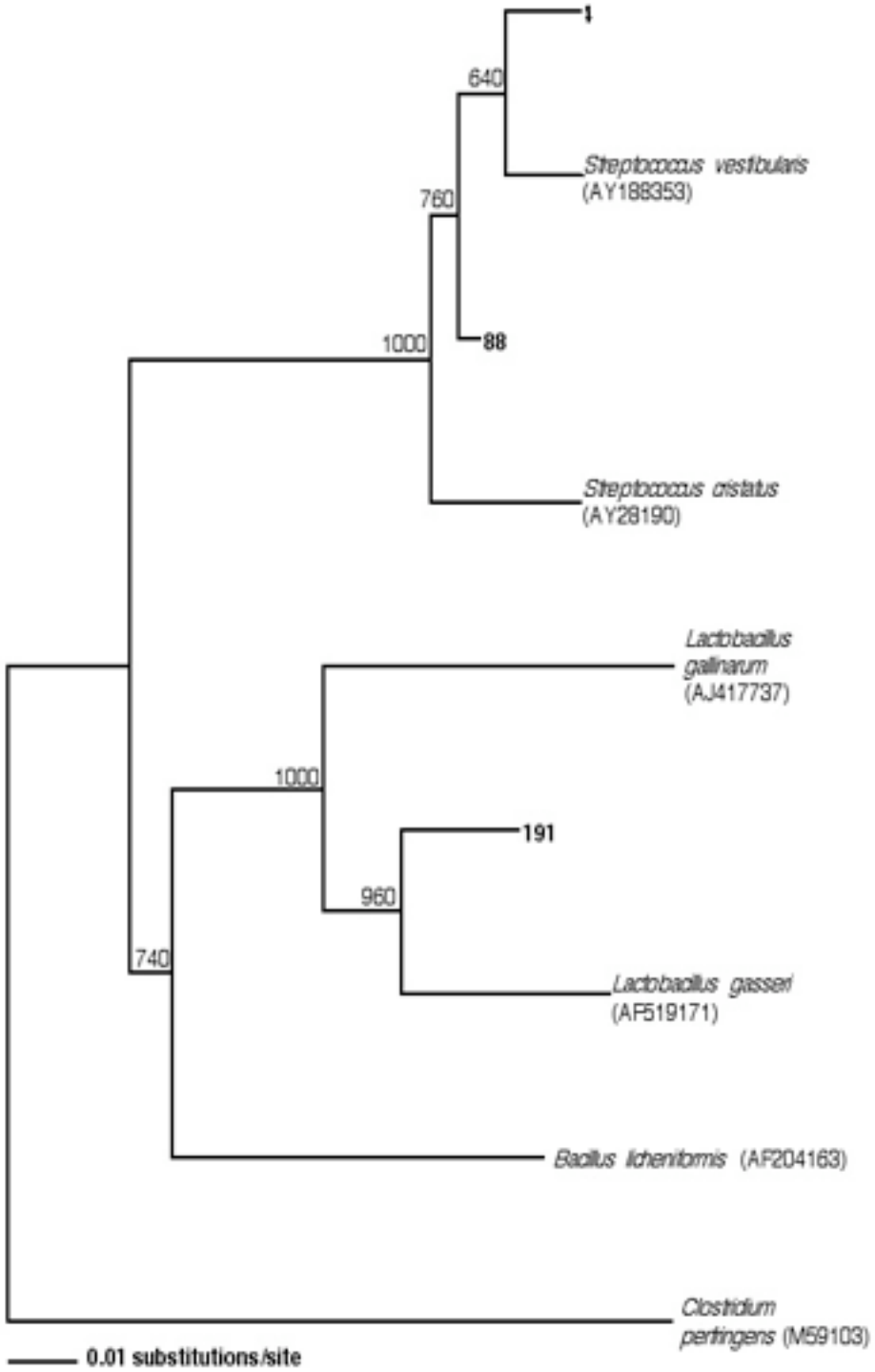
Figure 1: Typical RFLP analysis of cloned bacterial sequences from eastern bluebird feathers. The well on the far left is a 100 bp ladder (L), while the remaining lanes are individual sequences digested with the 4-base cutting restriction enzymes *Msp*I and *Hin*P1 I.

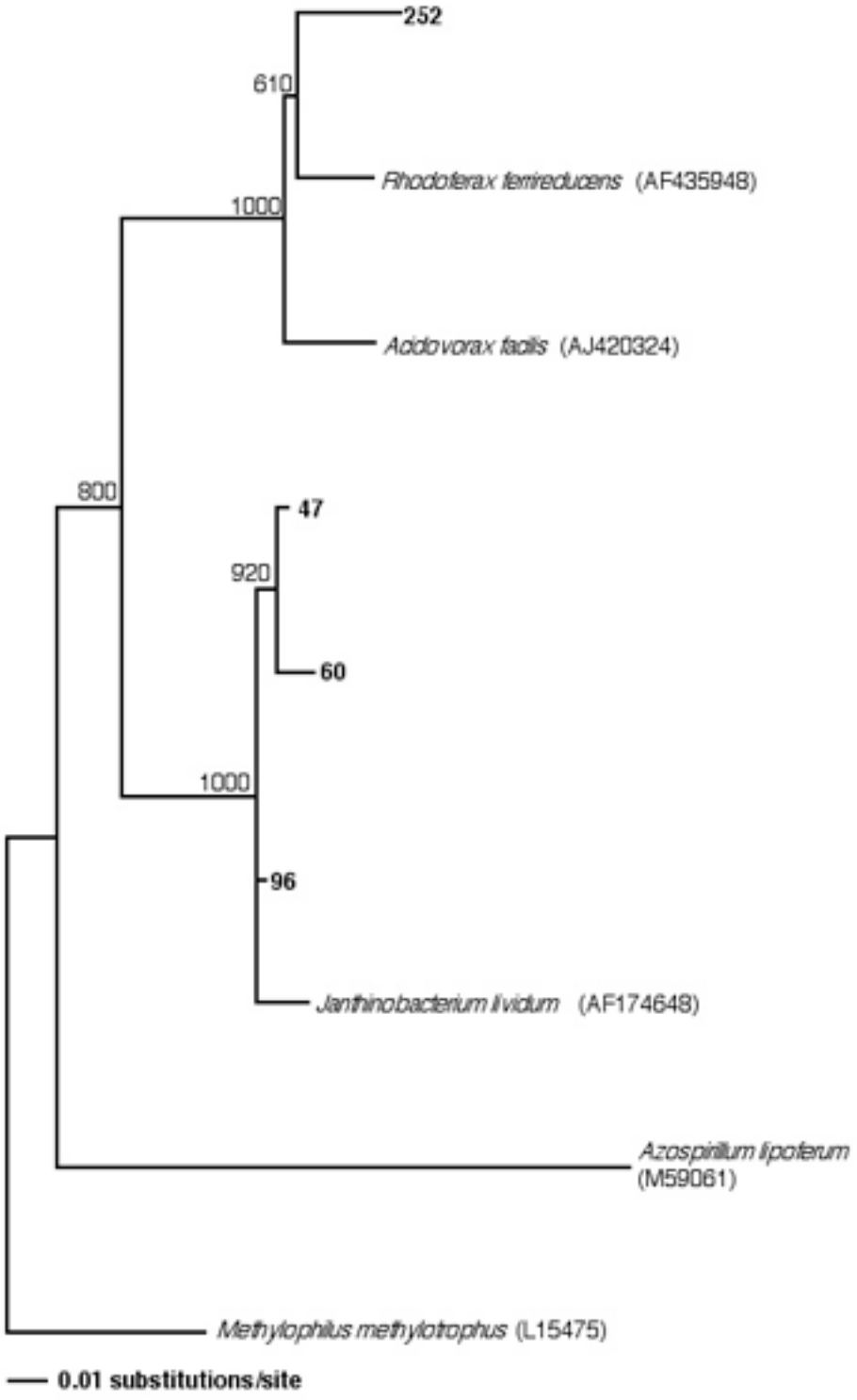
Figure 2: Neighbor-joining trees of unique cloned bacterial sequences from eastern bluebird feathers and related sequences from GenBank and the Ribosomal Database Project. A:γ-proteobacteria, B: β-proteobacteria, C: firmicutes. Bold letters indicate unique cloned sequences. Numbers close to the nodes represent bootstrap values obtained from 1000 bootstrap replicates.

L 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467

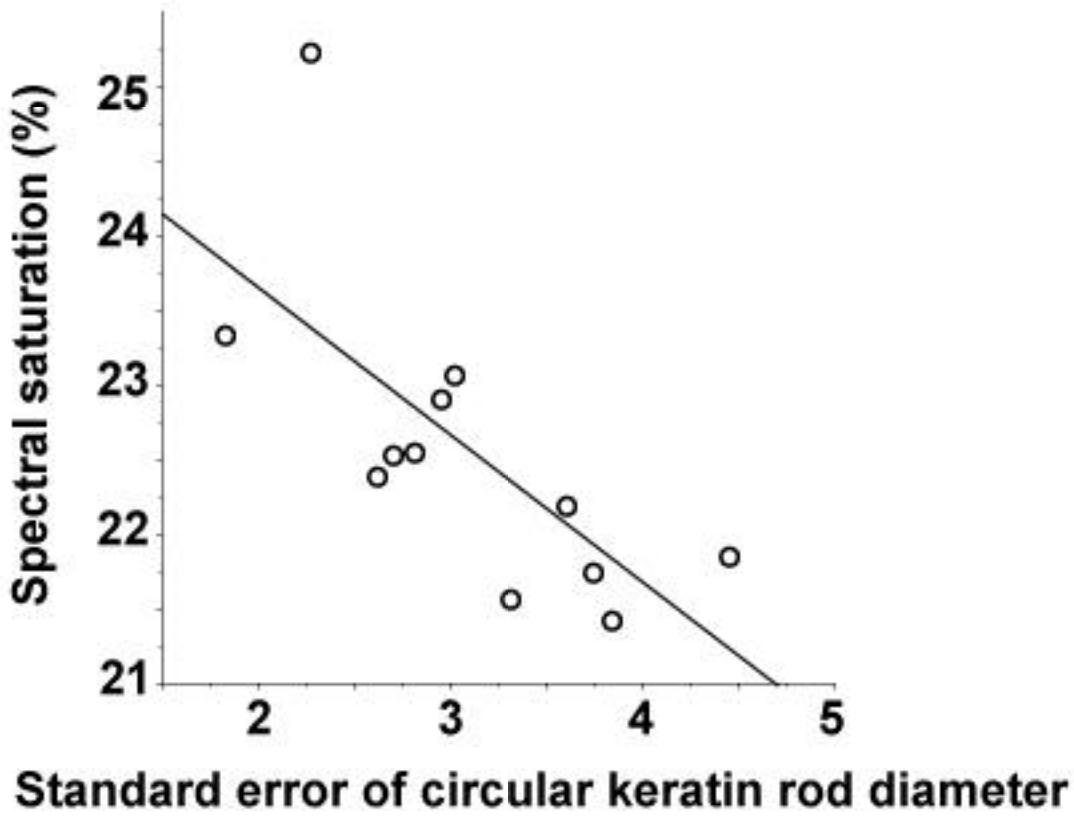



78
720
1000
Aeromonoas veronii (X60414)
Aeromonas hydrophila (X74676)
Pseudomonas lundensis (AB021395)
9
720
590
Pseudomonas fluorescens (AF094731)
840
73
1000
85
1000
15
2
1000
531
432
1000
Stenotrophomonas maltophila
(AF017749)
753
1000
Acinetobacter venetianus (AJ295007)
791
1000
1000
Ewingella americana (X88848)
1000
Yersinia pseudotuberculosis (AF36637)
Escherichia coli (X80724)
Vibrio cholerae (X74694)
0.01 substitutions/site


1
640
Streptococcus vestibularis
(AY188353)
760
88
1000
Streptococcus oristatus
(AY29190)
Lactobacillus
gallinarum
(AJ417737)
1000
191
960
740
Lactobacillus gasseri
(AF519171)
Bacillus licheniformis (AF204163)
Clostridium
perfringens (M59103)
0.01 substitutions/site


252
610
Rhodoferax fermentans (AF435948)
1000
Acidovorax facilis (AJ420324)
800
47
920
60
1000
96
Janthinobacterium lividum (AF174648)
Azospirillum lipoferum
(M59061)
Methylophilus methylotrophus (L15475)
0.01 substitutions/site

(b)


Spectral saturation (%)
25
24
23
22
21
2
3
4
5
Standard error of circular keratin rod diameter

Chapter 5

Chemical warfare?: Effects of uropygial oil on feather-degrading bacteria.

Abstract

Anti-microbial activity is a commonly suggested but rarely tested property of avian uropygial oil. Birds may defend themselves against feather-degrading and other potentially harmful bacteria using this oil. We preliminarily identified 13 bacterial isolates taken from the plumage of wild House Finches (*Carpodacus mexicanus*), measured bacterial production of the enzyme keratinase as an index of feather-degrading activity, and used the disc-diffusion method to test bacterial response to uropygial oil of House Finches. For comparison, we performed the same tests on a type strain of the known feather-degrading bacterium *Bacillus licheniformis*. Uropygial oil inhibited the growth of three strongly feather–degrading isolates (including *Bacillus licheniformis*), as well as one weakly feather-degrading isolate and one non-feather-degrading isolate. Uropygial oil appeared to enhance the growth of one weakly feather-degrading isolate. Growth of the remaining isoaltes was unaffected by uropygial oil. These results suggest that birds may defend themselves against some feather-degrading bacteria using uropygial oil.

The oily secretions of the uropygial gland of birds preserve feather flexibility, and provide an insulating and waterproofing layer when spread over the feathers (Jacob and Ziswiler 1982). The alkyl-substituted fatty acids and alcohols found in uropygial oil can retard the growth of bacteria and fungi (Jacob and Ziswiler 1982). For example, Jacob *et al*. (1997) showed that the fatty acid 3,7-dimethyloctan-1-ol found in Pelicaniform preen oil actively inhibited the growth of dermatophyte fungi. It has been postulated (Gill 1995; Jacob and Ziswiler 1982) that these secretions regulate the microbial flora on feathers, but this hypothesis remains virtually untested. Such regulation could be important in maintaining the integrity of feathers, as certain bacteria (e.g. Böckle *et al*. 1995; Burtt and Ichida 1999; Sangali and Brandelli 2000) and fungi (Santos *et al*. 1996) have been shown to degrade feathers *in vitro*. Microbial degradation of feathers on wild birds could decrease the insulation, lift (Burtt and Ichida 1999; Clayton 1999) and optical signaling that feathers provide. Feathers may also harbor opportunistic pathogens that could cause disease and infection (Scullion 1989). For these and other reasons, it may be important for birds to control their microbial flora.

The antimicrobial activity of uropygial oil has been tested only a few times. Baxter and Trotter (1969), Pugh and Evans (1970), and Pugh (1972) demonstrated that feather oils inhibited the growth of some keratinophilic fungi, but enhanced the growth of others. Bandyopadhyay and Bhattacharyya (1996) found that domestic fowl uropygial secretions enhanced the growth of the bacteria *Staphylococcus epidermidis*, *Streptomyces* spp. and *Proteus* sp., but inhibited the bacterium *Bacillus anthracis*. All of these bacterial isolates had been taken from the skin of these fowl. Thus, the effects of uropygial oil on microbial communities of birds appear to be complex. Uropygial oil

may promote the growth of mutualists that out-compete or otherwise exclude parasitic or pathogenic microbes (Pugh and Evans 1970).

As a first step towards understanding the effects of uropygial secretions on the bacteria of feathers, we preliminarily identified 13 isolates of bacteria from House Finches (*Carpodacus mexicanus*), measured the feather-degrading activity of each, and used the disc-diffusion assay (NCLLS 1997) to test the effect of House Finch uropygial oil on bacterial growth. For comparison, we also used the type strain *Bacillus licheniformis* O.W.U. 138B (ATCC # 55768). This bacterium is known to degrade feathers *in vitro*, and similar strains have been isolated from House Finches (Burtt and Ichida 1999).

Methods

Isolation and identification of bacteria

We captured 29 adult male House Finches in mist nets and feeder traps on the campus of Auburn University in Lee County, Alabama (32°35'N, 82°28'W) from April 1- July 1, 2002. Wearing latex gloves, we gently rubbed a BBL™ CultureSwab™ (Becton-Dickinson, Sparks, MD) dipped in sterile phosphate-buffered saline (PBS) on the breast, rump, and crown of the birds. The swabs were immediately refrigerated and then washed in 1 ml sterile PBS within 24 hours of collection. We pipetted 100 µl of inoculated PBS onto plates containing feather meal agar (FMA; Sangalli and Brandelli 2000;15 g l^{-1} feather meal, 0.5 g l^{-1} NaCl, 0.30 g l^{-1} K_2HPO_4, 0.40 g l^{-1} KH_2PO_4, and 15 g l^{-1} agar), a medium selective for feather-degrading bacteria (Sangalli and Brandelli 2000). We then incubated plates at 37° C for two weeks. We isolated several bacteria with distinctive colony morphology from FMA, re-streaked them on Tryptic Soy Agar (TSA,

Difco, New Jersey), a generalized medium, and then incubated them at 37° C for 48-72 hours until we could determine the purity of our cultures as evidenced by unique colony morphology characteristics.

We grew thirteen bacterial isolates with unique colony morphology overnight at 37° C in tryptose soy broth (Becton-Dickinson, Sparks, Maryland). We extracted DNA from each bacterium with the Instagene DNA purification matrix (Bio-Rad laboratories, Hercules, CA) as previously described (Pillai *et al*. 2001). We used primers 63F (5' CAGGCCTAACACATGCAAGTC 3') and 1389R (5'ACGGGCGGTGTGTACAAG 3'), corresponding to *Escherichia coli* 16SrDNA gene sequence in a 50 μl reaction volume according to the protocol of Osborn *et al*. (1999). The 16s rDNA PCR products were detected by electrophoresis in 2% agarose gels containing 0.6 ng/ml of ethidium bromide. Agarose gel electrophoresis was run for 3 h at 100V, and the gels were photographed under ultraviolet illumination using a digital image analysis system (ChemiImager, Alpha Innotech Corporation, San Leandro, CA).

Amplified PCR products from isolates were purified from agarose gels with a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). These purified gel products were sequenced at the Auburn University Genomics and Sequencing Laboratory using the same primers as above. Automated sequencing was done using the Big Dye Terminator cycle sequencing kit with an ABI Prism 3100 genetic analyzer and associated software (Applied Biosystems, Foster City, CA). Bacterial 16srDNA sequences were deposited in GenBank (accession numbers AY269864 – AY269876), and the BLAST algorithm was used to search for homologous sequences in the GenBank nr database (NCBI, Bethesda, Maryland). In addition to the isolates taken from House Finches, we

used *Bacillus licheniformis* strain O.W.U.138B (ATCC # 55768) as a positive control in our studies.

Measurement of keratinase

We tested for feather-degrading activity by measuring the quantity of keratinase produced by each bacterial isolate. This enzyme catalyzes the hydrolysis of keratin (Böckle *et al*. 1995; Santos *et al.* 1996) and is thus probably produced in proportion to a bacterium's feather-degrading activity. First, we washed House Finch rump feathers three times in distilled water, dried them overnight at 65° C and placed 5 mg of feathers in each of 45 separate tubes. To control for the possibility that differently-colored feathers may be differentially resistant to degradation, we only used feathers taken from birds whose rump hue scored between 9 and 11 (corresponding to an orange-red color) when measured on a Colortron™ portable spectrophotometer (Hill 1998). We autoclaved these feathers for 5 min at 120° C. This sterilization period was kept short to prevent breakdown of feather keratin before addition of the bacteria. We then added 5 ml of sterile PBS (pH 7.3) to each tube.

Colonies of each unique bacterial isolate were grown for 24 h on TSA and added to sterile PBS until a turbidity comparable to McFarland standard # 1.0 (equivalent to about 3 x 10^8 bacterial cells/ml) was reached. For each isolate, we inoculated three replicate tubes with 200 µl of suspension. These tubes were incubated at 37° C with agitation at 200 rpm (C24 Incubator Shaker, New Brunswick Scientific, Edison Park, NJ) for three weeks. To ensure that our samples were not contaminated, we streaked 10 µl of solution from each tube on TSA at the beginning of the experiment and at the end of each week (four times total) and compared colony morphology to our original plates. No

obvious contamination was detected. Following this incubation period, intact feathers were removed using sterile forceps and the remaining solution was filtered through 0.2 μm Durapore membranes (Millipore, Billerica, MA, USA).

Next, we measured keratinase concentration in these samples following the method of Santos *et al.* (1996). Briefly, we added 2 ml of filtrate from each sample to 4 mg of keratin azure (Sigma, St. Louis) and incubated them at 42º C with constant agitation for 21h. The release of the blue dye, indicating keratin hydrolysis, was measured using a spectrophotometer (Tecan Rainbow Therma, Tecan U.S., Research Triangle Park, NJ) at 595 nm. Results were expressed as mean units of keratinase per ml, with1 unit (U) of keratinase defined as the activity required for 1.0 A_{595} increase in 3 h incubation.

Effects of uropygial oil

We sacrificed 46 captive House Finches from Auburn, AL, immediately extirpated their uropygial glands and refrigerated them at 4º C for a maximum of one week. All birds were sacrificed under federal banding permit 21661 with Auburn University Institutional Animal Care and Use Committee approval. To ensure that captivity did not affect the properties of uropygial oil, we also sacrificed two wild-caught House Finches. After washing the surface of each gland three times with 100% ethanol, we used sterile forceps to squeeze oil onto sterile paper discs (diameter 3 mm; Becton-Dickinson, New Jersey). Because the oil was viscous and difficult to handle, it was not possible to precisely measure the amount placed on each disc. Therefore, we added oil to each side until it was entirely discolored. Most glands contained enough oil to saturate 3-4 paper discs.

These impregnated discs were sterilized under UV radiation for 30 minutes on each side. This procedure was fairly effective, as we only observed contamination (i.e. growth of bacteria around the disk different from that on the rest of the plate) on three out of 149 discs. The plates with these contaminated discs were discarded, and the tests were repeated.

Colonies of each unique bacterial isolate grown for 24 h on TSA were suspended in sterile PBS (pH 7.3) and adjusted to a turbidity comparable to McFarland standard # 0.5 (equivalent to 1.5 x 10^8 bacterial cells/ml). Mueller-Hinton agar (Difco, New Jersey) plates were then inoculated with these suspensions using sterile cotton swabs.

Each of the 14 bacterial isolates was then tested against oil from five different male and five different female birds (10 birds total). No bacterial isolate was tested against oil from the same bird twice. We placed paper discs saturated with oil from individual birds, as well as one blank control disc, on inoculated plates. No more than eight discs were placed on each plate. Plates were incubated at 37°C for 18 hours and were photographed using a digital image analysis system (ChemImager, Alpha Innotech Corporation, San Leandro, CA). We then measured the diameter of zones of inhibition using AlphaImager software.

Effects of UV sterilization

To ensure that the sterilization process did not affect the outcome of our tests, we repeated the disc diffusion assays described above without UV sterilization, using two individual birds for each isolate (14 isolates x two birds/isolate for a total of 28 disks). Although 54% (15/28) of the discs were clearly contaminated, the results we obtained were similar to those obtained using UV-sterilized discs (see table 1).

Results

Identification

We identified 13 bacterial isolates from swabs of House Finches, with 95-99% matches to known 16srDNA sequences in GenBank in all cases. Identification results are summarized in Table 1. These identifications are preliminary, and are considered accurate only to the genus level.

Keratinase production

Keratinase assay results are shown in Table 1. Isolate 1 produced a quantity of keratinase comparable to that of *Bacillus licheniformis*. Nine other isolates produced smaller amounts of keratinase, while three did not produce any detectable amount of keratinase.

Effects of uropygial oil

Bacteria grew normally around control discs, suggesting that they did not affect bacterial growth. Uropygial oil from every bird tested inhibited isolates 1, 2, 6 and 9, while oil from eight of ten birds inhibited *B. licheniformis* (Table 1). Based on the diameters of zones of inhibition, oil appeared to most strongly inhibit isolate 6. These results should be viewed with caution, however, as we did not control for the amount of oil placed on each disk. Uropygial oil did not affect any of the other isolates.

Oil appeared to enhance the growth of isolate 5, as bacterial growth was more dense in the zone surrounding the discs.

Effects of gender

Diameters of zones of inhibition were not significantly different between males and females for any isolate (all $P > 0.5$). Of the two samples that did not inhibit *B.*

licheniformis, one was a male and the other was a female. The inhibitory properties of this oil thus did not appear to vary with gender.

Effects of captivity

We tested uropygial oil from wild-caught birds against isolates 6 and 8 and *B. licheniformis* and found that, consistent with our results from captive birds, it inhibited the former two isolates but not the latter. Zones of inhibition were 12.32 ± 2.00 for isolate 6, 10.33 ± 1.33 for *B. licheniformis*, comparable to those for captive birds (see Table 1). Thus, captivity did not appear to affect the antimicrobial properties of this oil.

Discussion

Uropygial oil clearly inhibited several isolates, including three that strongly degraded feathers, but did not inhibit other isolates. The anti-microbial agent may be one of the many short-chain fatty acids and alcohols found in preen oil (Jacob and Zisweiler 1982; Jacob *et al*. 1997). Identifying these anti-microbial agents should be an exciting avenue for future research.

We predicted that growth of harmful bacteria would be inhibited whilst that of neutral or beneficial bacteria would either be unaffected or enhanced. At this point, however, too little data exist on the effects of bacteria on wild birds to test this prediction.

Of the type strain and isolates that were inhibited, three (*B. licheniformis*, isolates 1 and 2) produced large amounts of keratinase. The feather-degrading activity of these isolates has yet to be tested on live birds. The other two inhibited isolates were both of the genus *Staphylococcus*. *S. epidermidis* has also been associated with the disease bumblefoot in birds (Jennings 1954, Scullion 1989), and species of this genus are known to be opportunistic infectious agents (Keymer 1958a,b). However, these data are too

general to conclude that birds specifically defend themselves against *Staphylococcus*. Only the growth of isolate 5 was enhanced by uropygial oil and as far as we are aware, no data exist on the effects of bacteria of the genus *Micrococcus* on birds. No other isolates were affected by uropygial oil. Some *Bacillus* spp. are known to have detrimental effects on mammals (Parker 1984), although data are lacking for birds. *Pseudomonas* spp. have been associated with respiratory tract infections in ostriches (Momotani et al. 1995). *Rothia* spp. have been associated with infections in humans (Salomon and Prag 2002), but not in birds. Thus, at this point we can not say with certainty whether the inhibited bacteria are harmful or whether the non-inhibited bacteria are harmless. More research on the effects of bacteria on wild birds is critically needed.

The study of the interactions between birds and bacteria is barely in its infancy but already we can see the potential for such studies to help us better understand the life histories, ecology and physiology of birds and the distribution and abundance of microbes. Several fundamental lines of investigation are needed. We must determine the effects of isolated bacteria on wild birds. Once we have determined that an inhibited bacteria is harmful, for example, we can say with more certainty that the bird actively defends itself against it using uropygial oil. We can then begin testing the effectiveness of this defense through experimental manipulations. Ultimately, we may find evidence for the co-evolution of microbes or microbial community and the uropygial oil of their avian hosts. Our results here suggest that birds defend themselves against some bacteria using uropygial oil.

Acknowledgements

We thank K.L. Farmer for providing us with extirpated uropygial glands, and E.H. Burtt, Jr. and J. M. Ichida for providing cultures of *B. licheniformis*. S. R. Roberts gave methodological advice. K. L. Mills and E.L. Torstrick ably assisted with lab work. M. L. Beck, S.M. Doucet, A.M. Estes, K.L. Farmer, H.L. Mays,Jr., D.P. Mennill, and L. Siefferman provided helpful comments on the manuscript. This work was supported in part by NSF grants DEB-0077804, IBN-9722971, and ROI-AI49724 (to G.E.H.).

Figure legends

1) Disc diffusion assay tests of House Finch uropygial oil against an isolate of the bacterial genus *Staphylococcus* isolated from wild House Finches. Bacteria-free zones around the three discs impregnated with uropygial oil indicate inhibition of bacterial growth by the oil. The disc with no zone of inhibition is an unimpregnated control.

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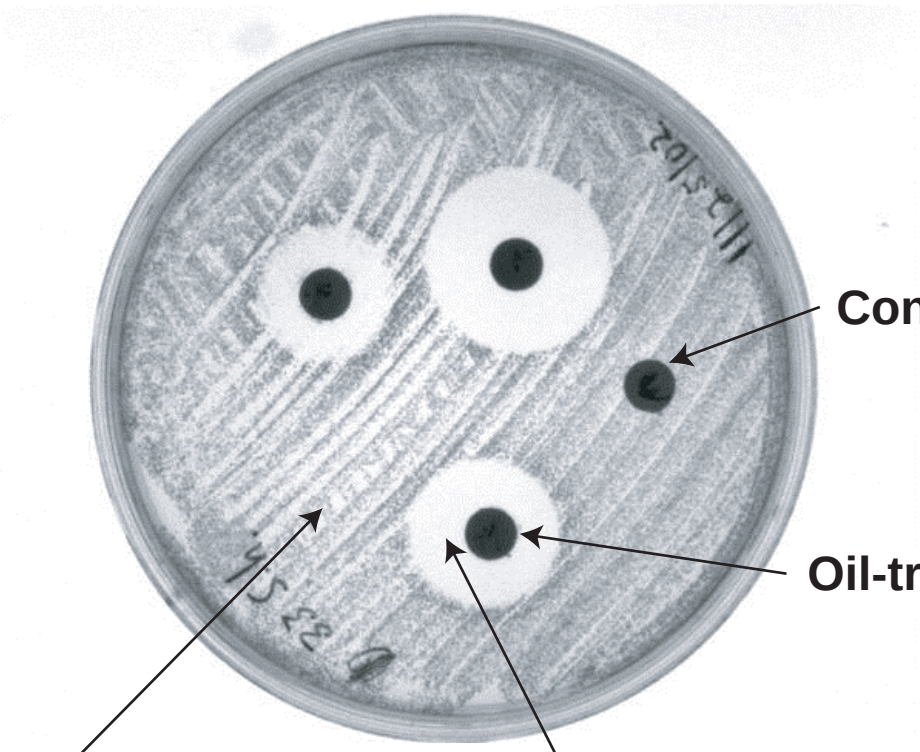
Table 1. Identification, keratinase production, and inhibition by House Finch uropygial oil of 13 bacteria isolated from the feathers of House Finches in Auburn, AL. *Bacillus licheniformis* O.W.U.138B (ATCC # 55768) is a type strain with known feather-degrading properties. The species in GENBANK with the closest DNA sequence to each isolate (as determined by the BLAST algorithm) is presented as a preliminary identification. Zone diameter was measured as the area of bacterial clearing around the disk impregnated with preen oil, with the exception of isolate five. Zone diameter for isolate five was measured as the area of enhanced bacterial growth around the disk. The final column contains results of inhibition tests in which the preen oil was not UV-sterilized before use.

.

Isolate #	Isolated from	Highest BLAST match	Base pairs matched	Keratinase production (U/ml x 10^{-3} ± 1 SE)	Zone diameter (mm ± 1 SE)	**No UV:** Zone diameter (mm ± 1 SE)
1	Rump	*Kocuria rhizophila*	567/595 (95%)	4.17 ± 0.45	12.20 ± 0.79	12.00 ± 1.41
2	Rump	*Bacillus* sp.	999/1009 (99%)	3.15 ± 0.53	10.28 ± 0.43	10.03 ± 0.71
3	Rump	*Pseudomonas stutzeri*	1004/1014 (99%)	2.87 ± 0.67	0.00	0.00
4	Rump	*Pseudomonas fulva*	1018/1028 (99%)	2.73 ± 0.32	0.00	0.00
5	Breast	*Micrococcus nishinomyaensis*	1039/1070 (97%)	2.49 ± 0.33	11.00 ± 1.66 (E)	10.8 ± 0.70 (E)
6	Breast	*Staphylococcus epidermidis*	982/1011 (97%)	2.30 ± 0.21	15.12 ± 3.05	13.46 ± 1.50
7	Breast	*Bacillus pumilus*	1039/1060 (98%)	1.58 ± 0.45	0.00	0.00
8	Breast	*Enterococcus fecalis*	1095/1116 (98%)	0.87 ± 0.15	0.00	0.00
9	Crown	*Staphylococcus hemolyticus*	1012/1022 (99%)	0.49 ± 0.30	10.91 ± 0.67	11.23 ± 0.98
10	Breast	*Staphylococcus hominis*	1001/1021 (98%)	0.20 ± 0.10	0.00	0.00
11	Breast	*Bacillus anthracis*	1010/1030 (98%)	0.00 ± 0.11	0.00	0.00
12	Rump	*Bacillus thuringiensis*	1012/1032 (98%)	0.00 ± 0.10	0.00	0.00
13	Breast	*Rothia amarae*	944/991 (95%)	0.00 ± 0.06	0.00	0.00
		Bacillus licheniformis O.W.U.138B	--	3.25 ± 0.30	10.78 ± 0.75	10.42 ± 0.99


Control disk
Oil-treated disk
Area of bacterial growth
Area of inhibited bacterial growth

Chapter 6

Bacteria as an agent for change in structural plumage color: correlational and experimental evidence.

Abstract

Recent studies have documented that bacteria with the ability to degrade keratin are present on wild bird feathers, and that uropygial oil inhibits their growth. These two findings suggest that birds may regulate the microbial flora on their feathers, perhaps to prevent feather degradation. If bacteria on feathers degrade coloured barbs and barbules then plumage coloration could reveal bacterial infestation. We used field- and lab-based methods to test the hypothesis that male eastern bluebirds (*Sialia sialis*) with brighter blue structural coloration of feathers were better able to regulate their microbial flora than duller males. Contrary to predictions, when we sampled male bluebirds in the field, individuals with brighter color had higher bacterial loads than duller individuals. In the lab, we tested whether bacteria could directly alter feather color through degradation. We found that keratinolytic bacteria increased the brightness and purity of structural color, but decreased UV chroma. This change in spectral properties of feathers likely occurs through degradation of the cortex and spongy layer of structurally colored barbs. These data suggest that bacteria can alter plumage color, perhaps partially accounting for the change in structural plumage color across time observed in studies of wild birds. Furthermore, males may gain some benefit in sexual attractiveness from wear caused by bacteria, suggesting that bright structural feather color may function as an indicator of a handicap, or may not be a reliable indicator of male quality.

Indicator models of sexual selection propose that ornamental traits signal aspects of male quality (reviewed in Andersson 1994). One important aspect of quality for virtually all animals is the degree to which an individual is parasitized. High-quality males should have fewer parasites than low-quality males, and if ornaments are signals of quality, males with few parasites should be more highly ornamented than males with many parasites. Females should prefer to mate with males with low parasite loads to avoid parasite transfer (Borgia 1986; Freeland 1976; Hilgarth 1996), gain greater parental care (Hamilton 1990; Milinski and Bakker 1990; Møller 1990), or to pass on genes for parasite resistance (Hamilton and Zuk 1982).

Hundreds of studies have tested these hypotheses, most of them focusing on protozoan or metazoan parasites of the blood, gut, or integument (reviewed in Møller *et al*. 1999). Bacteria are ubiquitous and they sometimes have deleterious effects on organisms, yet only a few studies have documented associations between bacterial infection and sexually selected traits (Brawner *et al*. 2000; Hill *et al. in press*; Hill and Farmer *in press*). Clearly more studies that include bacteria are needed.

Feather degradation may be one effect of bacterial infestation. Feather-degrading bacteria could negatively affect their hosts by digesting feathers, thereby decreasing the thermoregulation and protection from other bacterial infections provided by feathers (Burtt and Ichida 1999; Clayton 1999; Muza *et al*. 2000). Like virtually all organisms, birds coexist with a diverse bacterial flora. Only a small subset of these will be detrimental to the bird, and an even smaller subset will cause damage to feathers.

Burtt and Ichida (1999) isolated the feather-degrading bacterium *Bacillus licheniformis* from 8% of 1588 wild birds surveyed. Other authors have isolated bacteria

with keratin-degrading properties in the genera *Vibrio* (Sangali and Brandelli 2000), *Fervidobacterium* (Friedrich and Antranikian 1996), *Pseudomonas* (Shawkey *et al.* 2003a), Arthrobacter (Lucas *et al.* 2003), and *Streptomyces* (Böckle *et al.*,1995; Ichida *et al.* 2001; Mukhopadyay and Chandra 1990; Noval and Nickerson 1959. Furthermore, a recent study by Moureau *et al.* (in press) demonstrated that birds with experimentally enlarged broods have higher densities of bacteria on their feathers. Preen oil applied to feathers (Bandyopadhyay and Bhattacharyya 1996; Shawkey *et al.* 2003) may inhibit bacterial growth, so these results suggest a trade-off between energy spent on foraging or reproduction and that devoted to preening or other sanitation behaviors.

A relationship between feather color and bacterial load on feathers could thus arise in at least two ways. First, color might signal overall quality, and higher quality birds might be better able to allocate energy to sanitation behaviors that reduce bacterial load than poor quality birds. If this hypothesis is true, then we would predict that birds with more elaborate ornamentation would have lower abundances of feather-degrading bacteria. Second, bacteria may directly affect plumage color by wearing down the structures involved in the production of color (Shawkey and Hill 2004). This possibility seems particularly likely in the case of structural plumage color, which is based on the reflection of light from complexly arranged tissues (reviewed in Prum 1999). Örnborg *et al.* (2002) recently showed that the structurally-based UV-blue crown color of blue tits (*Parus caeruleus*) becomes brighter and less UV-reflective over the course of the breeding season, suggesting that wear on feathers, perhaps partially caused by feather-degrading bacteria, may change their reflective properties. In this case, we would predict a positive relationship between feather bacteria and the brightness of structural

coloration, and a negative relationship between feather bacteria and UV reflectance. Bacteria exist in complex communities (Brock *et al*. 1994) and some bacteria that do not degrade feathers themselves may be required for the action of feather-degrading bacteria (Moureau *et al*. *in press*). We must therefore consider both the overall bacterial community as well as the feather-degrading subset of this community when examining this hypothesis.

The eastern bluebird (*Sialia sialis*) is an excellent model organism on which to test these hypotheses. Structural UV-blue plumage color of bluebirds appears to be sexually selected; males with brighter blue structural plumage color have greater competitive ability than duller males (Siefferman and Hill *in press*), and males with brighter structural and melanin breast coloration have higher reproductive success than duller males (Siefferman and Hill 2003). Furthermore, the anatomical basis of variation in the coloration of bluebird feathers is fairly well understood (Shawkey *et al*. 2003b; Shawkey *et al*. *in press* a), allowing us to identify specific mechanisms associated with bacterially mediated changes in plumage color.

We used both field and lab-based methods to test whether bacteria cause a change in plumage coloration or are just correlated with such a change. First, we trapped adult male bluebirds and measured the abundance of total and feather-degrading bacteria on their feathers. We measured the color of the feathers of these birds with a spectrometer. We then correlated measures of bacterial abundance to plumage color. In the lab, we further tested our second hypothesis by inoculating groups of feathers with feather-degrading bacteria and examining changes in color and microstructure after a brief incubation period.

METHODS

Sampling

In June 2002 and March 2003 we captured adult male eastern bluebirds in mist nets and box traps on the campus of Auburn University in Lee County, Alabama, USA (32°35'N, 82°28'W). Wearing sterile latex gloves, we gently rubbed a BBL™ CultureSwab™ (Becton-Dickinson, Sparks, MD) dipped in sterile phosphate-buffered saline (PBS) on their structurally-colored rump feathers. Following bacterial sampling, we pulled feathers from the rump of each bird for color analysis. Swabs were immediately refrigerated and then washed in 1 ml sterile PBS within 4 hours of collection, and feathers were stored in small manila envelopes in a climate-controlled room until the time of color analysis.

Media

We used two types of media for quantification of bacteria. Tryptic soy agar (TSA) (Difco, Detroit, MI) is a rich medium that supports a wide variety of microorganisms, and its use enabled us to quantify overall bacterial load, including most feather-degrading and other bacterial flora. We sampled for the feather-degrading subset of these bacteria by using feather meal agar (FMA, Sangali and Brandelli 2000), containing 15 g l^{-1} feather meal, 0.5 g l^{-1} NaCl, 0.30 g l^{-1} K_2HPO_4, 0.40 g l^{-1} KH_2PO_4, and 15 g l^{-1} agar. FMA was used to estimate specifically feather-degrading bacterial load, as it contains feather keratin as its sole source of carbon (Sangali and Brandelli 2000). In another study, we found that almost all bacterial isolates taken from this media produced keratinase, an enzyme that catalyzes the hydrolysis of keratin (Shawkey *et al.* 2003a). Therefore, most bacteria growing on it should be able to digest keratin and could be

considered putatively "feather degrading." Both media types contained 100 μg/ ml of cycloheximide to inhibit fungal growth (Smit *et al.* 2001).

We plated 100μl of our inoculated samples on TSA and FMA and incubated the plates at 37° C. TSA plates were removed after two days, while FMA plates were removed after 14 days. The faster growth of microorganisms on TSA than on FMA necessitated this difference in incubation time, as colonies began to merge, and hence became uncountable, after 48 hours on TSA. FMA plates were incubated longer as colonies could be clearly distinguished only after two weeks. The number of visible colony forming units (CFUs) on each plate was counted for each medium type as estimates of total and feather degrading bacterial load (total plate count; see Bettin *et al.* 1994; Brock *et al.* 1994; Hambreaus *et al.* 1990; Miller *et al.* 1994). All counts were performed without knowledge of the bird's color scores.

For color analysis, we taped feathers in stacks of five 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 fiber optic probe at a 90° angle 5mm from the feather surface, creating a measurement area of 2mm 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 haphazardly chosen points on each sample.

From these reflectance spectra, we calculated colour variables for each sample. We restricted these indices to wavelengths between 300 and 700 nm, as evidence suggests that passerine birds are sensitive to ultraviolet (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 1999, Keyser and Hill 1999, 2000). Brightness, the mean of reflectances from 300-700 nm, is a measure of the total amount of light reflected by the feathers (Andersson 1999; Endler 1990). UV-violet (UV-V) chroma is the percentage of total light reflected in the range of 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 *et al.* 2001).

Experimental methods

We performed experimental inoculation of bluebird feathers twice with nearly identical methodology. In both cases, we arbitrarily picked five rump feathers from each of 40 different males from a large collection of feathers in our lab and measured them using a spectrometer as above. We then divided these feathers into experimental and control groups of twenty individuals each. In the first experiment, we washed all feathers in ethanol twice and allowed them to dry in a sterile hood. In the second experiment, we sterilized feathers with 10kgy of γ-radiation at the Auburn University Space Research Center.

To simulate a humid environment, we constructed simple humidified chambers in Petri dishes using layers of sterile wet and dry blotter paper and plastic. Sterile circular pieces of blotter paper were saturated with sterile water and placed on the outermost position of the top and bottom portions of the dishes. Between these wet layers, we placed two pieces of dry sterile blotter paper sandwiching two pieces of sterile plastic.

In a previous study, we had identified a large number of bacterial isolates from bluebird feathers (Shawkey *et al. in press* b). We tested a subset of these bacteria for keratinolytic activity following the methods of Shawkey *et al*. (2003a) and chose for our experimental treatment a highly keratinolytic bacterium, identified as closely related to *Bacillus pumilus* by 16S rDNA sequence analysis (Shawkey *et al. in press* b). Colonies of this bacterium were grown for 24 h on TSA and added to sterile PBS until a turbidity comparable to McFarland standard # 1.0 (equivalent to about 3 x 10^8 bacterial cells/ml) was reached. We then inoculated feathers with 500 µl of either this bacterial solution (experimental group) or sterile PBS (control group), patted them with sterile tissues to absorb excess moisture, and placed them between the two plastic layers of the humidified chambers.

We incubated these chambers at 37° C for 72 hours, then removed them and placed the feathers in new sterile Petri dishes. We washed feathers in ethanol and allowed them to air dry in a sterile hood. After drying for 24 hours, all feathers were taped to black construction paper and measured with the spectrometer as above.

Microscopy

To determine how bacteria damaged feathers and whether this damage caused change in feather color, we examined feathers from our inoculation experiment with a scanning electron microscope. We mounted one feather from each experimental sample and one feather from five control samples in experiment two on stubs (Ted Pella, Redding, CA) using carbon tape (Ted Pella, Redding, CA), sputter-coated them with gold on a Electron Microscopy Sciences sputter coater (Hatfield, PA, USA) and viewed them on a Zeiss DSM 930 Scanning Electron Microscope (Oberkochen, Germany). We took

five consecutive photos of barbs at 500x magnification, moving clockwise from the distal end of the central rachis of each feather. We defined damaged surface area as any disruption in the integrity of the barb's surface (see figure 4). We then measured total surface area and damaged surface area in NIH Image v. 1.62 (available for download at http://rsb.info.nih.gov/nih-image). We divided damaged surface area by total surface area as a relative index of feather damage

Analyses

All analyses were performed on SPSS v.10 for Macintosh (SPSS 2002). Percentage data were arcsine transformed, and bacterial-count data were log transformed. We correlated TSA and FMA plate counts with color variables using Spearman's rank correlation test because our data were not normally distributed. We used paired t-tests to compare color of experimental and control groups before and after manipulation, and created a proportional "color change" variable for each color measurement by subtracting each post-experiment color measurement from the corresponding pre-experiment measurement and dividing by this original measurement. Finally, we correlated these color change variables with amount of feather damage using Pearson's correlation test. All tests were two-tailed.

RESULTS

In June 2002, total bacterial load and brightness were significantly positively correlated (Spearman rank correlation, $r_s = 0.504$, $p = 0.010$, $n = 25$, figure 1, table 1), but feather-degrading bacterial load and brightness were not significantly correlated ($r_s = 0.164$, $p = 0.456$, $n = 25$, figure 1, table 1). No other variables were significantly correlated (all $p > 0.4$, table 1). When we replicated this experiment in March 2003 we

found similar patterns. Again, total bacterial load and brightness were significantly positively correlated (Spearman rank correlation, $r_s = 0.654$, $p = 0.029$, $n = 11$, figure 1, table 1), but feather-degrading bacterial load and brightness were not significantly correlated ($r_s = 0.422$, $p = 0.196$, $n = 11$, figure 1, table 1). No other variables were significantly correlated (all $p > 0.4$, table 1).

In the first humidified chamber experiment in which feathers were cleaned with ethanol, the color of the control group had significantly lower UV-V chroma following sham manipulation ($t = 5.07$, $p > 0.001$, table 2, figure 2, 3). Additionally, control feathers tended to have higher spectral saturation ($t = -1.74$, $p = 0.10$, table 2, figure 2, 3), and brightness ($t = -1.65$, $p = 0.12$, table 2, figure 2, 3) following treatment, but these differences were not significant. The experimental feathers that had been inoculated with *B. pumilus* had significantly lower UV-V chroma ($t = 2.59$, $p = 0.02$, table 2, figure 2, 3), but higher spectral saturation ($t = -3.31$, $p = 0.008$, table 2, figure 2, 3) and brightness ($t = -5.03$, $p = 0.001$, table 2, figure 2, 3). Hue did not change significantly in either the control or the experimental groups (both $p \geq 0.19$, table 2, figure 2, 3).

In the second humidified chamber experiment in which feathers were sterilized using γ- radiation, the control group did not change significantly in any color measurement following sham manipulation (all $p > 0.4$; table 2, figure 2, 3). This difference in color change in the control feathers in the two experiments was probably due to differences in the effectiveness of sterilization. In the first experiment, ethanol probably failed to kill all of the bacteria on the feathers before manipulation. In the second experiment, γ-radiation killed all bacteria on the feathers.

The experimental group had significantly lower UV-V chroma ($t = 3.56$, $p = 0.002$, table 2, figure 2, 3), but higher spectral saturation ($t = -2.50$, $p = 0.02$, table 2, figure 2, 3) and brightness ($t = -2.95$, $p= 0.008$, table 2, figure 2, 3), following incubation with feather-degrading bacteria. Hue did not change significantly in either the control or experimental group (both $p >0.12$, table 2, figure 2, 3).

Bacteria appeared to damage feathers primarily by stripping away the cortex and burrowing into the spongy layer, sometimes leading to breakage of barbs and barbules (Figure 4). As expected, feathers in the experimental group were significantly more damaged than those in the control groups (Mann-Whitney $U = 5.00$, $Z = -3.06$, $p = 0.001$, figure 4,5). Within our experimental group, change in UV-V chroma during the experiment was positively correlated with feather damage, such that feathers that were heavily damaged during the experiment lost more UV-V reflectance than those that were lightly damaged ($r = 0.52$, $p = 0.02$, figure 6). Feather damage was not significantly correlated with change in any other color variable (brightness, $r = 0.12$, $p = 0.62$; spectral saturation, $r = 0.20$, $p = 0.41$; hue, $r = -0.33$, $p = 0.16$; figure 6).

DISCUSSION

We found that male bluebirds with brighter structural color had a higher total bacterial load on their feathers than males with duller plumage. These patterns do not support the hypothesis that bright coloration indicates overall ability to regulate microbial flora. This result was unexpected, given the greater competitive ability of brighter versus duller male eastern bluebirds (Siefferman and Hill *in press*). We expected males with more elaborate plumage coloration to have fewer bacteria in their feathers.

This negative relationship between bacterial load and color could have arisen in a number of ways. First, bright, dominant male eastern bluebirds may allocate more time to display and territorial defense then to sanitation behaviors, leading to an increase in bacterial numbers on feathers. This possibility could be tested by recording the time spent preening by dull versus bright birds. Other studies have shown a negative link between social dominance and condition (e.g Poiani *et al.* 2000) suggesting that dominance may entail health costs. Similarly, starlings *Sturnus vulgaris* with experimentally increased reproductive workloads had more numerically abundant bacterial communities on their feathers than those with reduced workloads (Moureau *et al. in press*), perhaps because they devote less time to sanitation behavior. Brighter bluebirds may also raise a larger number of young than duller birds, and thus invest more energy in reproduction, leaving less for self-maintenance (Siefferman and Hill 2003). However, we found a similar relationship between brightness and bacterial load both before egg-laying and after the completion of the last clutch, so this explanation seems unlikely.

Second, some of these bacteria may have unknown beneficial effects (Lombardo *et al.* 1999), and brighter birds may be better able to acquire these bacteria than duller birds. Testing this hypothesis will first require extensive research into the diverse effects that bacteria may have on birds, as our knowledge of these effects is largely limited to a small number of pathogenic bacteria at present (Brawner *et al.* 2000; Hill *et al. in press;* Hill and Farmer *in press).* Such studies would greatly enhance our understanding of the complex interactions between birds and microbes, but are beyond the scope of this paper.

Finally, bacteria may directly alter plumage color (Shawkey and Hill 2004). If brighter males have more bacteria because bacteria affect feathers in a way that increases brightness, we would expect to see a stronger correlation between feather-degrading bacterial load and color than between overall bacterial load and color. We observed the opposite- there was a strong relationship between feather color and overall bacterial load but not feather color and feather-degrading bacterial load. These results remain puzzling, but bacteria live in complex communities, and even bacteria that are not able to break down keratin themselves may play a role in feather degradation (Moureau *et al. in press*). Thus, entire communities of microbes may be needed for feather breakdown in the wild. Additionally, certain keratinolytic bacteria may be more effective at breaking down feathers than others, but may not be numerically dominant. The relationship between these important kertainolytic bacteria and plumage color may have been swamped in our analysis.

We directly tested the hypothesis that bacteria can directly change feather color in a series of lab experiments. These experiments provided direct evidence that feather-degrading bacteria are able to alter structural plumage color. In our first experimental trial in which feathers were cleaned using ethanol, the color of both control and experimental groups decreased in UV-V reflectance, and non-significant increases in brightness and spectral saturation in the color of the control group mirrored significant increases in the experimental group. When we repeated the experiment using γ-sterilization rather than ethanol washing, however, the color of the control group was completely unaffected by time in the humidified chambers, while the color of the experimental group was again brighter and more spectrally pure, but less UV-V reflective. Change in feather color

following sham treatment in the first experimental trial was likely caused by incomplete sterilization of the feathers. γ-sterilization effectively kills 100% of bacteria (Roberts 1985), while ethanol cleaning is not as reliable (Brock *et al*. 1994). Thus, the change in color of the control group in the first trial was probably caused by the action of residual bacteria. These experiments show the strong effects of a large dose of keratinolytic bacteria on feathers as well as the more subtle but easily detected effect of even a small dose of bacteria (those left alive after the ethanol wash) on feather color.

Örnborg *et al*. (2002) noted that UV-V chroma declined while brightness increased through the breeding season. Feather color changed in a nearly identical manner during our experiment, suggesting that these patterns in the field may be partially attributable to bacterial degradation. It is worth noting, however, that our inoculation of feathers with a single bacterial strain was a highly simplified test that did not account for the synergistic or antagonistic effects of other bacteria on feathers. Degradation of feathers may occur quite differently under natural conditions with the complex bacterial communities that appear to exist on feathers (Shawkey *et al*. *in press* b, Moureau *et al*. *in press*). These differences may partially explain why we found a relationship between brightness and bacterial load in the field, but not between UV-V chroma and bacterial load. Future studies should inoculate entire communities of bacteria on feathers.

The decrease in UV-V chroma appears to be caused by the smaller increase in reflectance in the UV range than in the visible range that results from bacterial degradation (see figure 2). The anatomical explanation for this difference probably involves changes in the cortex and spongy layer. As described previously (Shawkey *et al*. 2003b), the color-producing spongy layer of eastern bluebird feather barbs lies beneath a

keratin cortex and above a layer of melanin granules surrounding large central vacuoles. In a previous study (Shawkey *et al*. in press a), we showed that birds with more UV-reflective plumage had thicker spongy layer. In this study, we found that bacteria tended to peel away the cortex and spongy layer. This destruction of the spongy layer could decrease the amount of ultraviolet light reflected from the barbs. Indeed, we found a significant relationship between degree of damage to barbs and change in chroma. The loss of light-absorbing cortex (Finger 1995) may cause overall reflectance to increase (Shawkey *et al. in press* a). Additionally, the increased irregularity of the cortex surface caused by bacterial degradation may increase the amount of light scattered incoherently, increasing achromatic brightness (Bradbury and Vehrenkamp 1998). Bacteria may have similar effects on color resulting from melanin- and carotenoid- deposition. All feather barbs and barbules have an outer keratin cortex layer, and an increased "roughness" in this layer could increase the brightness of these feathers.

It is clear from our results that feather-degrading bacteria can directly alter structural plumage color, and this result is completely novel and potentially important for sexual signaling. A number of studies have shown evidence that females of some bird species prefer males with more UV-reflective feathers (Andersson *et al*. 1998; Sheldon *et al*. 1998). Other studies have found evidence that brightness and UV-V chroma of structural coloration signals the resource-holding potential of males (Keyser and Hill 1999; Siefferman and Hill *in press*). Thus, if bacteria change the color of feathers in wild birds, they may play an important role in the evolution of the signaling function of structural color.

These results seem to represent an evolutionary paradox. Higher chroma and brightness and more UV-shifted hue signals better condition and greater resource holding potential in male eastern bluebirds (Siefferman and Hill *in press*). Yet, it is the brightest males that have the highest bacterial loads. If the sexual attractiveness or dominance of male bluebirds increases with bacterial damage, bluebirds may purposely acquire or maintain bacteria. Extensive feather damage, however, will clearly have detrimental effects as thermoregulatory and flight efficiencies are reduced (Clayton 1999, Burtt and Ichida 1999). This paradox may be explained in at least two different ways. First, an increase in brightness from bacterial damage may be offset by a concurrent decrease in UV-V chroma. High brightness and UV-V chroma both appear to make male bluebirds more attractive (Siefferman and Hill 2003, Siefferman and Hill *in press*). Therefore, offsetting changes in these two variables may eliminate any benefits gained from bacterial damage. To test this idea we will need to determine the relative importance of brightness and UV-V chroma to sexual selection in bluebirds. Second, UV-blue structural color may serve as an indicator of a handicap (Zahavi 1975). High quality bluebirds may advertise their ability to survive despite feathers degraded by bacteria. To test this idea we will need to disentangle "natural" color, i.e. the color of new-grown feathers, from "induced" color, i.e. color after abrasion and degradation. This model will work most clearly if damage causes color to shift to values outside of those seen in feathers without wear. In this way females will be able to distinguish naturally bright males from induced bright males. Alternatively, bluebirds may vary little in their color soon after molt, but vary extensively following several months of damage before the breeding season, and females may choose this induced color. This type of gene-

environment interaction in sexual ornaments may prove to be a fascinating avenue for future research.

These data also leave open the possibility that brightness is not a reliable signal of male quality. Birds may be naturally bright or they may become bright by feather damage. If the system is open to "cheaters", then the signal content of the color display will be eroded and both males and females should lose interest in it over evolutionary time (Hill 1994).

This study shows that bacteria on the surface of feathers may play an important, hitherto unexamined role in the maintenance and evolution of structural plumage color signaling. The potential for interactions between microbes and feathers is vast, and should be explored through correlative and experimental studies of not only structural, but also melanin and carotenoid-based coloration.

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FIGURE LEGENDS

Figure 1: The brightness of rump feathers in relation to total and feather-degrading bacterial loads among eastern bluebirds in Auburn, AL in June 2002 and March 2003. N = 25 in 2002 and n = 11 in 2003.

Figure 2: Reflectance spectra of feathers before and after inoculation with either the feather-degrading bacterium *B. pumilus* or a sham control. In the first experiment, feathers were washed with ethanol before inoculation, while in the second experiment feathers were sterilized with γ-radiation. The dark lines represent groups before treatment while the light lines represent the same groups after treatment. N = 20 in all cases.

Figure 3: Boxplots of color variables measured before and after inoculation with either the feather-degrading bacterium *B. pumilus* or a sham control. In the first experiment, feathers were washed with ethanol before inoculation, while in the second experiment feathers were sterilized with γ-radiation. The dark boxes represent groups before treatment while the light boxes represent the same groups after treatment. The line within each box represents the median number of barbules, the upper and lower borders of each box are the 25^{th} and 75^{th} percentiles and the lower and upper bars are the 10^{th} and 90^{th} percentiles. Asterisks indicate significant ($p<0.05$) differences. N = 20 in all cases.

Figure 4: Boxplot of percentage of feather barb surface area that was damaged following inoculation with either the feather-degrading bacterium *B. pumilus* or a sham control. The line within each box represents the median number of barbules, the upper and lower borders of each box are the 25^{th} and 75^{th} percentiles and the lower and upper bars are the 10^{th} and 90^{th} percentiles.

Figure 5: Scanning electron micrograph (500x) of the outer surface of a feather barb experimentally damaged by the feather-degrading bacterium *B. pumilus* and an undamaged feather barb. SL = spongy layer, C = cortex, B = bacterial cells.

Figure 6: Scatterplots of change in color variables vs. relative surface area of damaged feather barbs following inoculation with the feather-degrading bacterium *B. pumilus.* N = 20 in all cases.

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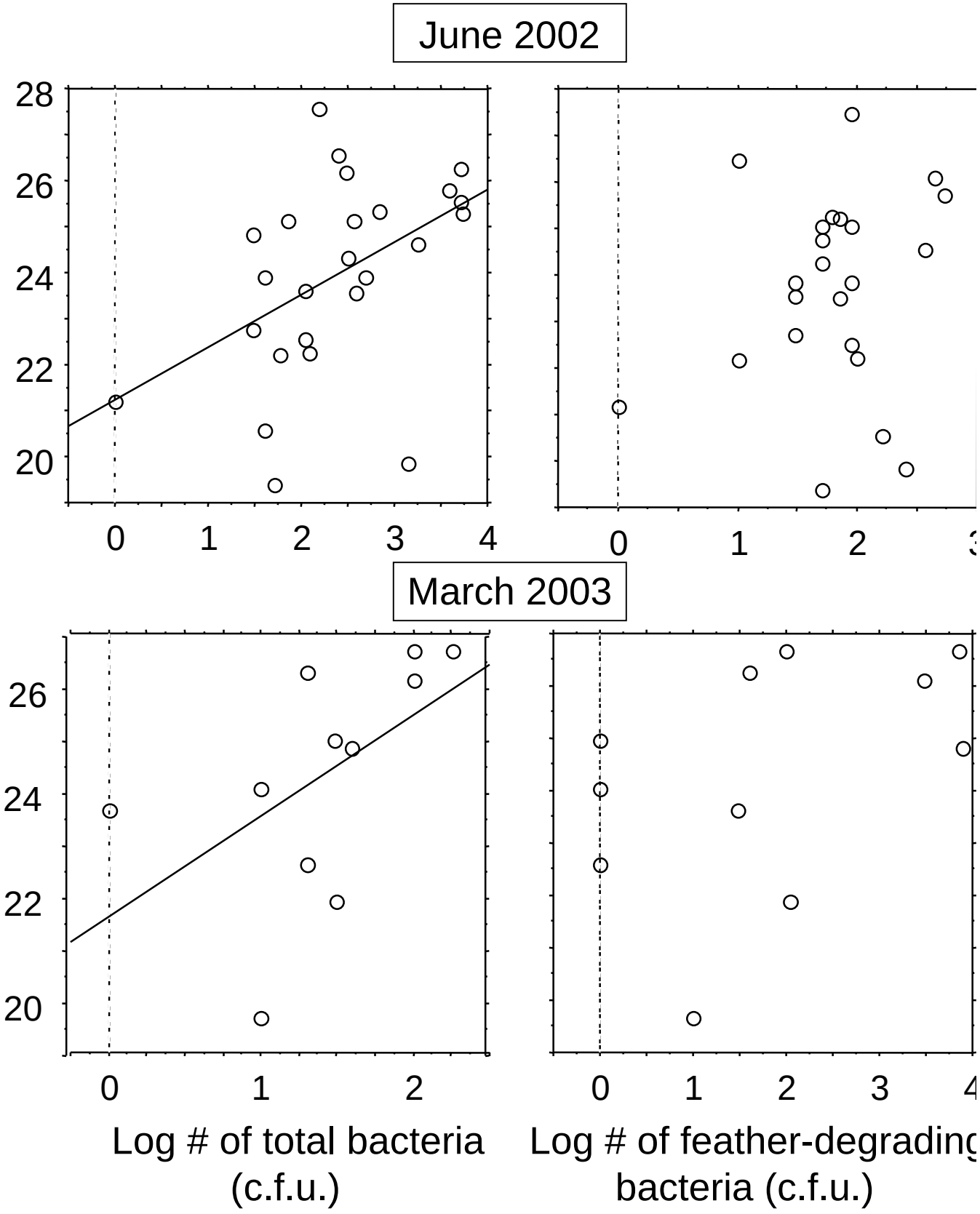
Table 1. Spearman rank correlations of total and feather-degrading bacterial loads with color variables of rump feathers of eastern bluebirds. N = 25 for June 2002 and N = 11 for March 2003. Asterisks indicate significant ($p<0.05$) correlations.

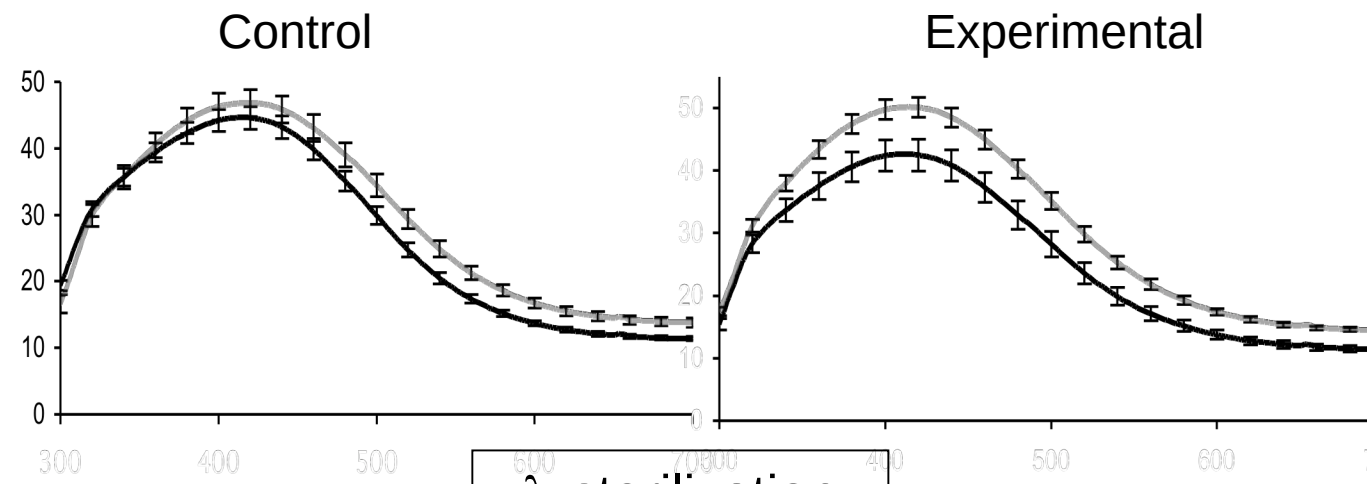
	Sampling period	Hue (nm)	UV-V chroma (%)	Brightness (%)	Spectral saturation (%)
Total bacterial load	June 2002	0.05	-0.02	0.50*	0.03
Feather-degrading bacterial load	June 2002	-0.05	-0.13	0.16	0.10
Total bacterial load	March 2003	0.01	0.04	0.65*	0.11
Feather-degrading bacterial load	March 2003	0.00	0.14	0.20	0.16

Table 2. Comparison of the color of eastern bluebird feathers before and after incubation in humidified chambers with either the feather-degrading bacterium *Bacillus pumilus* or a sham control.

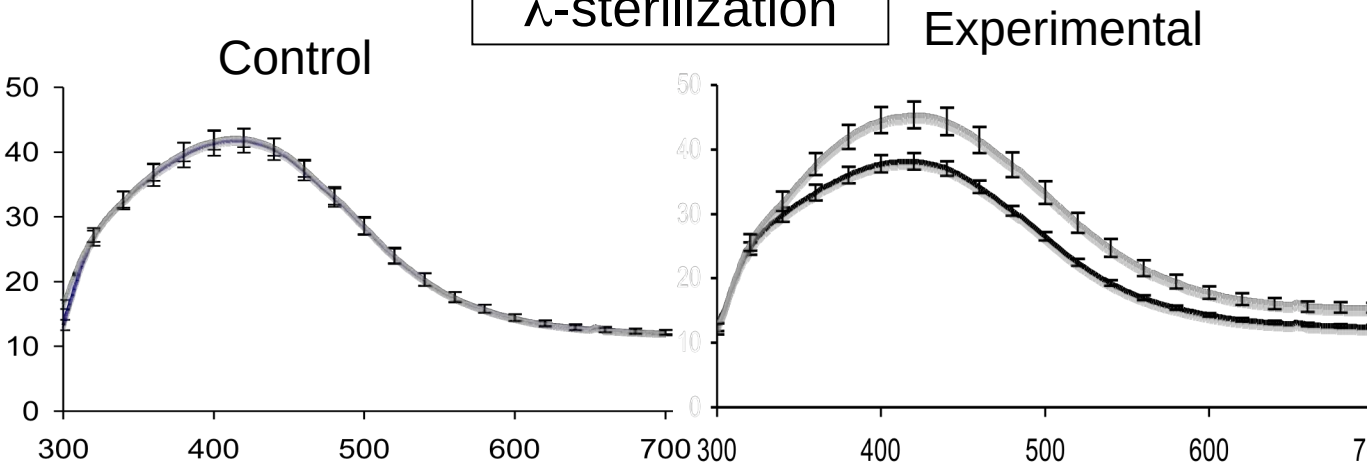
	Sterilization method	Control before manipulation (±1 S.E.)	Control after manipulation (±1 S.E.)	*t*	*p*	Experimental before manipulation (±1 S.E.)	Experimental after manipulation (±1 S.E.)	*t*	*p*
Hue (nm)	Ethanol	416.08 ± 2.43	419.06 ± 2.97	-1.36	0.19	416.52 ± 3.12	416.46 ± 3.57	0.02	0.98
Brightness (%)	Ethanol	27.02 ± 0.93	29.45 ± 1.33	-1.65	0.12	24.79 ± 0.90	30.45 ± 1.32	-5.03	<0.01
UV-V chroma (%)	Ethanol	41.87 ± 0.49	38.97 ± 0.69	5.07	<0.01	41.34 ± 0.85	39.49 ± 0.72	2.59	0.02
Spectral saturation (%)	Ethanol	22.38 ± 0.91	24.28 ± 0.06	-1.74	0.10	21.26 ± 0.95	25.12 ± 1.36	-3.31	<0.01
Hue (nm)	γ-radiation	419.91 ± 3.10	419.61 ± 3.28	0.31	0.64	419.01 ± 2.52	423.65 ± 3.30	-1.65	0.12
Brightness (%)	γ-radiation	25.69 ± 0.85	26.00 ± 0.95	-0.30	0.77	24.11 ± 0.56	28.14 ± 1.42	-2.95	<0.01
UV-V chroma (%)	γ-radiation	40.00 ± 1.00	40.00 ± 1.00	-0.72	0.48	39.00 ± 0.64	36.00 ± 0.81	3.56	<0.01
Spectral saturation (%)	γ-radiation	20.31 ± 0.70	20.56 ± 0.89	-0.26	0.80	18.55 ± 0.60	21.33 ± 1.66	-2.5	0.02


June 2002
28
26
24
22
20
0
1
2
3
4
March 2003
26
24
22
20
0
1
2
Log # of total bacteria (c.f.u.)
0
1
2
3
Log # of feather-degrading bacteria (c.f.u.)

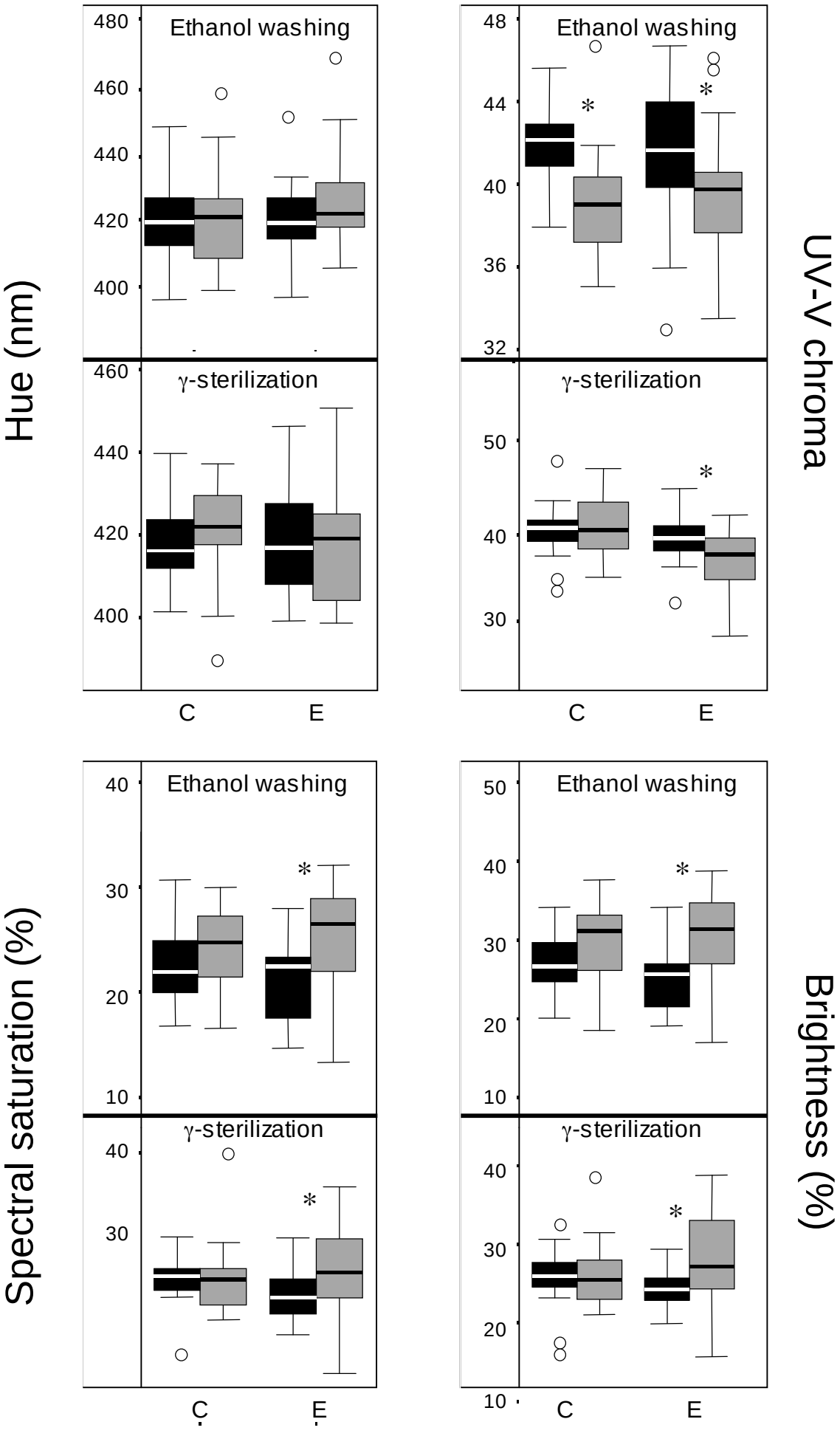
Ethanol washing

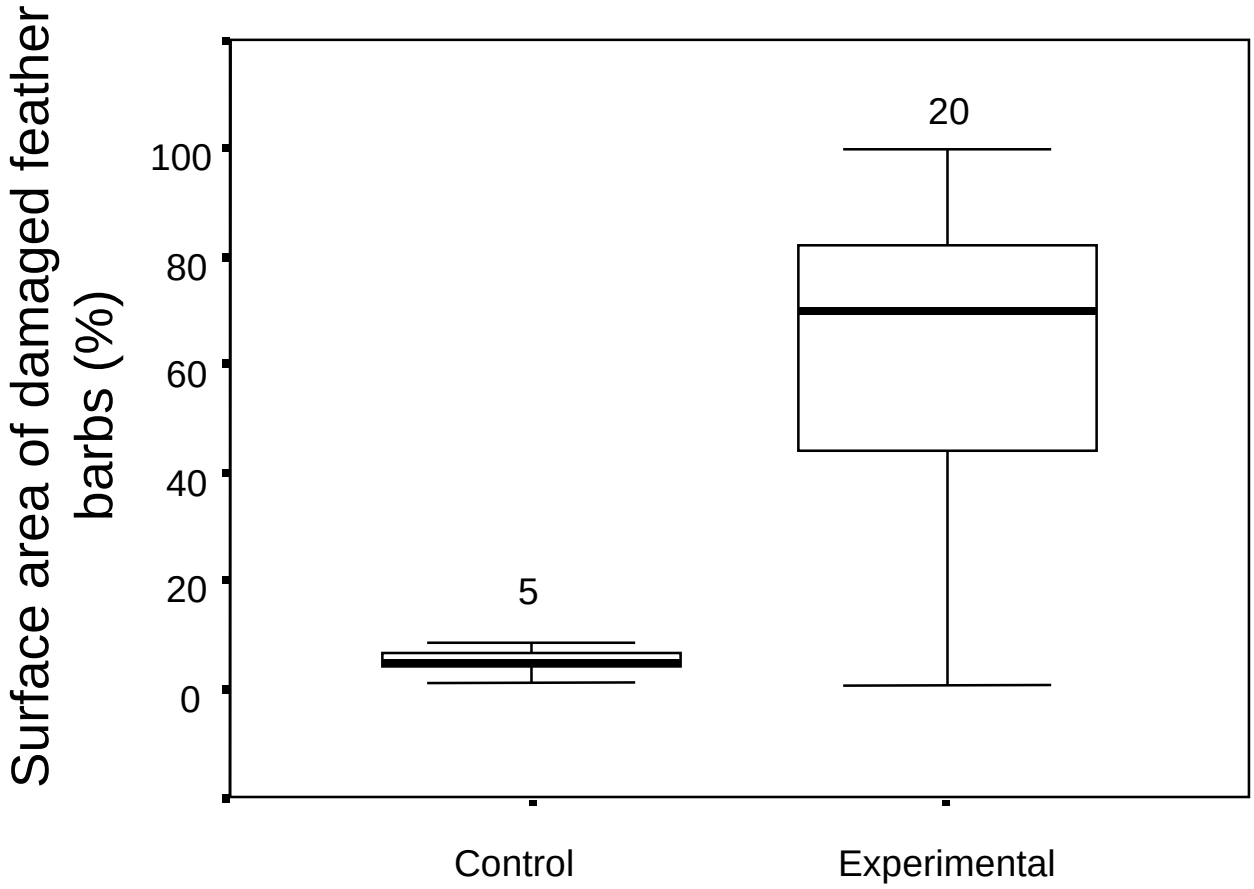


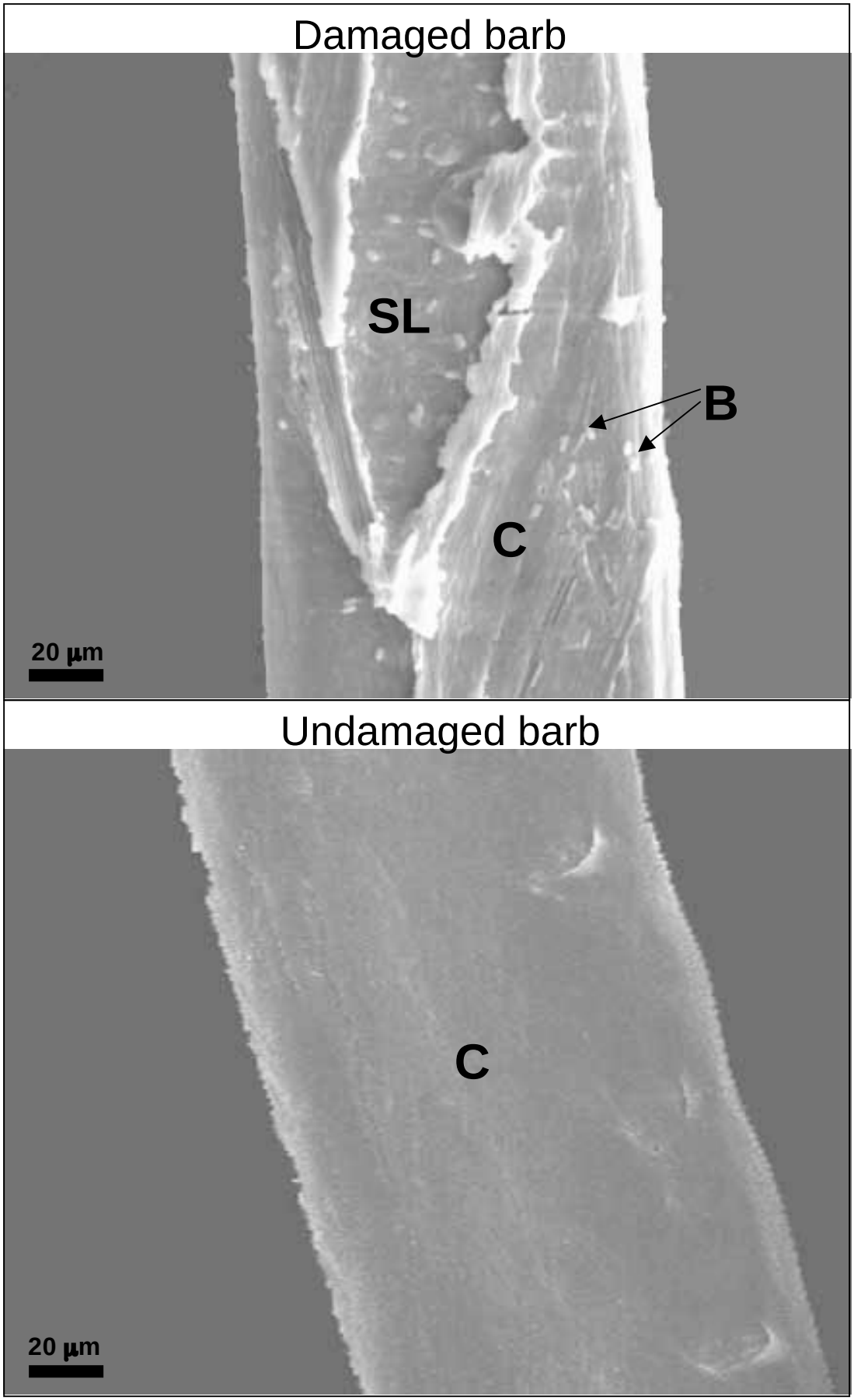
λ-sterilization

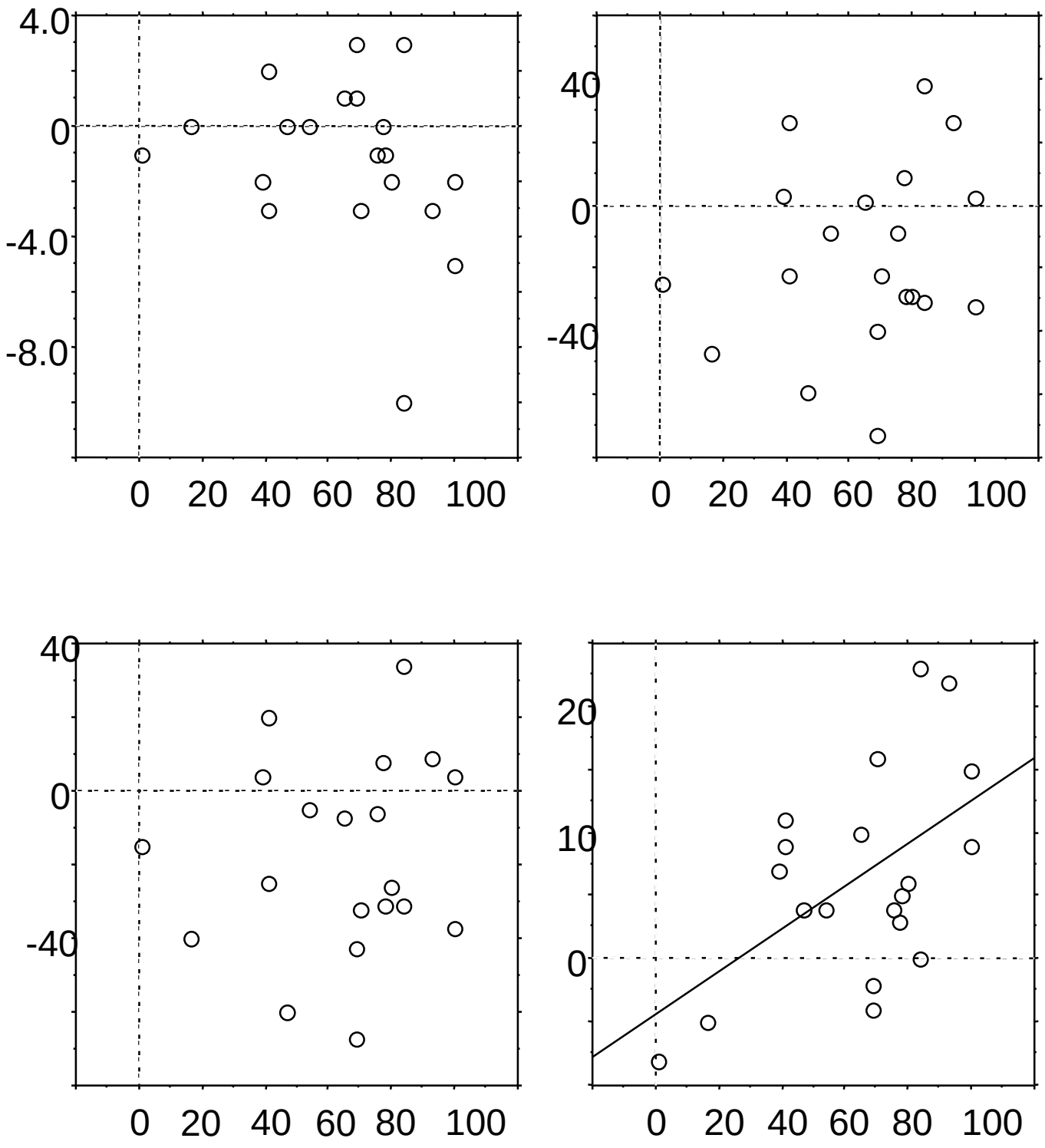


Wavelength (nm)


Hue (nm)
UV-V chroma
Spectral saturation (%)
Brightness (%)
Ethanol washing
γ-sterilization
C
E


Surface area of damaged feather barbs (%)
100
80
60
40
20
0
20
5
Control
Experimental


Damaged barb
SL
B
C
20 µm
Undamaged barb
C
20 µm


4.0
0
-4.0
-8.0
40
0
-40
40
0
-40
20
10
0
0 20 40 60 80 100
Damaged surface area of feather barbs (%)