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Bacteria as an Agent for Change in Structural Plumage Color: Correlational and Experimental Evidence

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Abstract: Recent studies have documented that a diverse assemblage of bacteria is present on the feathers of wild birds and that uropygial oil affects these bacteria in diverse ways. These findings suggest that birds may regulate the microbial flora on their feathers. Birds may directly inhibit the growth of harmful microbes or promote the growth of other harmless microbes that competitively exclude them. If keratinolytic (i.e., feather-degrading) bacteria degrade colored feathers, then plumage coloration could reveal the ability of individual birds to regulate microbial flora. We used field- and lab-based methods to test whether male eastern bluebirds (Sialia sialis) with brighter blue structural plumage coloration were better able to regulate their microbial flora than duller males. When we sampled 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. We found that keratinolytic bacteria increased the brightness and purity, decreased the ultraviolet chroma, and did not affect the hue of structural color. This change in spectral properties of feathers may occur through degradation of the cortex and spongy layer of structurally colored barbs. These data suggest that bacteria can alter structural plumage color through degradation.

Keywords: feather-degrading bacteria, plumage color, structural color, sexual selection, Sialia sialis.

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Bacteria and Plumage Color

Figure 1: Brightness of rump feathers in relation to total and keratinolytic colony forming unit (c.f.u.) abundances among eastern bluebirds in Auburn, Alabama, in June 2002 (n = 25) and March 2003 (n = 11).

Table 1: Spearman rank correlations of general and feather-degrading bacterial loads with color variables of rump feathers of eastern bluebirds

<table>
<thead>
<tr>
<th>Sampling period</th>
<th>Hue (nm)</th>
<th>UV-V chroma (%)</th>
<th>Brightness (%)</th>
<th>Spectral saturation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>General bacterial load:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>June 2002</td>
<td>.05</td>
<td>-.02</td>
<td>.50*</td>
<td>.03</td>
</tr>
<tr>
<td>March 2003</td>
<td>.01</td>
<td>.04</td>
<td>.65*</td>
<td>.11</td>
</tr>
<tr>
<td>Feather-degrading bacterial load:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>June 2002</td>
<td>-.05</td>
<td>-.13</td>
<td>.16</td>
<td>.10</td>
</tr>
<tr>
<td>March 2003</td>
<td>.00</td>
<td>.14</td>
<td>.20</td>
<td>.16</td>
</tr>
</tbody>
</table>

Note: For June 2002, n = 25, and for March 2003, n = 11. UV-V = ultraviolet-violet.

* Significant correlations at P < .05.
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 keratinolytic bacteria and the brightness of structural coloration and a negative relationship between keratinolytic bacteria and UV reflectance.

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 2003), 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, 2003a), allowing us to identify specific mechanisms associated with bacterially mediated changes in plumage color.

We used both correlational and experimental approaches to test whether bacteria cause a change in plumage coloration or are just correlated with such a change. First, we trapped adult male bluebirds, quantified the coloration of their feathers, and measured the abundance of total and keratinolytic bacteria on their feathers. We then correlated measures of bacterial abundance to plumage color. In the lab, we further tested for an effect of bacteria on structural coloration by inoculating groups of feathers with keratinolytic 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 (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 h of collection, and feathers were stored in small manila envelopes in a climate-controlled room (approximately 21°C) 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 keratinolytic and other bacterial flora. We sampled for the keratinolytic 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 K₂HPO₄, 0.40 g L⁻¹ KH₂PO₄, and 15 g L⁻¹ agar. FMA was used to specifically estimate keratinolytic bacterial abundance because 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 keratinolytic. Both media types contained 100 μg mL⁻¹ of cycloheximide to inhibit fungal growth (Smit et al. 2001).

We plated 100 μL of our inoculated samples on TSA and FMA and incubated the plates at 37°C. TSA plates were removed after 2 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 h on TSA. FMA plates were incubated longer because colonies could be clearly distinguished only after 2 weeks. The number of visible colony forming units (CFUs) on each plate was counted for each medium type as an estimate of general and keratinolytic bacterial load (total plate count; see Hambraeus et al. 1990; Bettin et al. 1994; Brock et al. 1994; Miller et al. 1994).

**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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control before incubation</th>
<th>Control after incubation</th>
<th>Experimental before incubation</th>
<th>Experimental after incubation</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hue (nm)</td>
<td>419.91 ± 3.10</td>
<td>419.61 ± 3.28</td>
<td>.31</td>
<td>419.01 ± 2.52</td>
<td>423.65 ± 3.30</td>
<td>1.65</td>
</tr>
<tr>
<td>Brightness (%)</td>
<td>25.69 ± .85</td>
<td>26.00 ± .95</td>
<td>−.30</td>
<td>24.11 ± .56</td>
<td>28.14 ± 1.42</td>
<td>−2.95</td>
</tr>
<tr>
<td>UV-V chroma (%)</td>
<td>4.00 ± 1.00</td>
<td>4.00 ± 1.00</td>
<td>−.72</td>
<td>3.90 ± .64</td>
<td>3.60 ± .81</td>
<td>3.56</td>
</tr>
<tr>
<td>Spectral saturation (%)</td>
<td>20.31 ± .70</td>
<td>20.56 ± .89</td>
<td>−.26</td>
<td>18.55 ± .60</td>
<td>21.33 ± 1.66</td>
<td>−2.5</td>
</tr>
</tbody>
</table>

Note: Control and experimental values are shown ±1 SE; t and P values are from paired t-tests comparing the same feathers before and after incubation.

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**References**


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**Bacteria and Plumage Color**

Figure 2: Reflectance spectra (±1 SE) of feathers before and after inoculation with either the keratinolytic bacterium *Bacillus pumilus* or a sham control. The dark lines represent groups before treatment, while the light lines represent the same groups after treatment; *n* = 20 in all cases.

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. Blue and gray portions of each feather were aligned with one another so that no mixing of the two colors occurred. This arrangement was similar to that found on the living bird. We recorded spectral data from the blue portion of these feather stacks with an Ocean Optics (Dunedin, FL) S2000 spectrometer (range 250–880 nm). Using a probe holder (RPH-1; Ocean Optics) with matte black interior that excluded ambient light, we held a bifurcated 1-μ fiber-optic probe at a 90° angle 5 mm from the feather surface, creating a measurement area 2 mm in diameter. The reading area was illuminated by both a UV (D-2000 deuterium bulb; Ocean Optics) 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 (Ocean Optics) to record and average 20 spectra sequentially, and we recorded and averaged measurements from five haphazardly chosen points on each sample.

From these reflectance spectra, we calculated color variables for each sample. We restricted these indexes to wavelengths between 300 and 700 nm because evidence suggests that passerine birds are sensitive to UV wavelengths (300–400 nm; Cuthill et al. 2000) and that 700 nm is the upper limit of the vertebrate visual system (Jacobs 1981). The wavelength of maximum reflectance was used as an index of hue, the principal color reflected by the feathers (e.g., Andersson 1999; Keyser and Hill 1999). Brightness, the mean of reflectances from 300 to 700 nm, is a measure of the total amount of light reflected by the feathers (Endler 1990; Andersson 1999). The 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 color purity (Pryke et al. 2001).

**Experimental Methods**

We performed experimental inoculation of bluebird feathers. 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 20 individuals each. We sterilized feathers with 10 kGy of γ-radiation at the Auburn University Space Research Center. This sterilization method effectively kills almost all (>99.9%) microbes (including fungi) on any surface (Roberts 1985; Silverman 1991).

To simulate a humid environment, we constructed simple humidified chambers in petri dishes by 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. 2005a). We tested a subset of these bacteria for keratinolytic activity following the methods of Shawkey et al.
Figure 3: Box plots of color variables measured before and after inoculation with either the keratinolytic bacterium Bacillus pumilus or a sham control. 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 color variable, the upper and lower borders of each box are the twenty-fifth and seventy-fifth percentiles, and the lower and upper bars are the tenth and ninetieth percentiles. Asterisks indicate significant (P < .05) difference; n = 20 in all cases.

(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. 2005a). Colonies of this bacterium were grown for 24 h on TSA and were added to sterile PBS until a turbidity comparable to McFarland standard 1.0 (equivalent to about 3 × 10⁸ bacterial cells mL⁻¹) 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 h and 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 h, 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 (SEM). We mounted one feather from each experimental sample and one feather from five control samples on stubs (Ted Pella, Redding, CA) using carbon tape (Ted Pella), sputter-coated them with gold on an Electron Microscopy Sciences sputter coater (Hatfield, PA) and viewed them on a Zeiss DSM 930 SEM (Oberkochen, Germany). We took five consecutive photos of barbs at × 500 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 fig. 4). We then measured total surface area and damaged surface area in NIH Image, version 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, version 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 the color of experimental and control groups before and after manipulation and then created a proportional “color change” variable for each color measurement by subtracting each postexperiment color measurement from the corresponding preexperiment measurement and dividing by this original measurement. Finally, we correlated these color change variables with the amount of feather damage using Pearson’s correlation test. All tests were two tailed.

Results

In June 2002, overall CFU abundance and brightness were significantly positively correlated (Spearman rank correlation, r = 0.504, P = .010, n = 25; fig. 1; table 1), but keratinolytic CFU abundance and brightness were not significantly correlated (r = 0.164, P = .356, n = 25; table 1). No other variables were significantly correlated (all P > .4; table 1). When we replicated this experiment in March 2003, we found similar patterns. Again, overall CFU abundance and brightness were significantly positively correlated (Spearman rank correlation, r = 0.654, P = .029, n = 11; fig. 1; table 1), but keratinolytic CFU abundance and brightness were not significantly correlated.
Variables were significantly correlated (all $p < 0.05$; table 1). Figure 4: Box plot of percentage of feather barb surface area that was damaged following inoculation with either the keratinolytic bacterium *Bacillus pumilus* or a sham control. The line within each box represents the median percentage of damaged area, the upper and lower borders of each box are the twenty-fifth and seventy-fifth percentiles, and the lower and upper bars are the tenth and ninetieth percentiles.

In the humidified chamber experiment, color measurements of the control group did not change significantly following sham manipulation (all $P > 0.4$; table 2; figs. 2, 3). The experimental group had significantly higher brightness ($t = 8.95$, $P = 0.008$; table 2; figs. 2, 3) and spectral saturation ($t = 2.50$, $P = 0.02$; table 2; figs. 2, 3) and significantly lower UV-V chroma ($t = 3.56$, $P = 0.002$; table 2; figs. 2, 3) following incubation with keratinolytic bacteria. Hue did not change significantly ($P > 0.12$; table 2; figs. 2, 3).

Bacteria appeared to damage feathers primarily by thinning the cortex and breaking barbs and barbules (fig. 4). As expected, feathers in the experimental group were significantly more damaged than those in the control groups (Mann-Whitney $U = 5.00$, $Z = -3.06$, $P = 0.001$; figs. 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 ($r = 0.52$, $P = 0.02$; fig. 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: $r = -0.33$, $P = 0.16$; fig. 6).

**Discussion**

We found that male bluebirds with brighter structural color had a higher overall CFU abundance on their feathers than males with duller plumage. These patterns did not support the hypothesis that bright coloration indicates overall ability to inhibit microbial growth. This positive 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 comparing 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, European starlings *Sturnus vulgaris* with experimentally increased reproductive workloads had more numerically abundant bacterial assemblages on their feathers than starlings with reduced workloads (Lucas et al. 2005), perhaps because they devoted 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 be beneficial (Lombardo et al. 1999), and brighter birds may be better able to acquire or enhance the growth of these bacteria than duller birds. In particular, some bacteria may competitively exclude the growth of pathogenic bacteria, as the normal microflora does on human skin (Tannock 1995). Testing these hypotheses will first require research into the effects that different species of bacteria have on birds both alone and in assemblages. At present, our knowledge of these effects is largely limited to a small number of pathogenic bacteria (reviewed in Charlton 2000). Such studies would greatly enhance our understanding of the complex interactions between birds and microbes but are beyond the scope of this article.

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 keratinolytic CFU abundance and color than between overall CFU abundance and color. We observed the opposite: there was a strong relationship between feather color and overall bacterial load but not feather color and keratinolytic CFU abundance. 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 (Lucas et al. 2005). Thus, entire assemblages of microbes may be needed for feather breakdown in the wild. Additionally, certain keratinolytic bacteria may be more ef-
Bacterial degradation appears to strongly increase overall brightness of feathers. As figure 2 illustrates, the change in reflectance over most wavelengths (excluding from 300 to 320 nm) of the experimental feathers following inoculation is higher than that of the controls. This increase, equivalent to multiplying the reflectance values of the experimental feathers before inoculation by a constant >1, probably explains the changes in other color variables. Multiplication of the large reflectance values around the peak wavelength results in a greater increase than at other wavelengths and thus heightened saturation. Because these peak wavelengths are outside of the UV, reflectance values within the UV increase proportionally less, causing decreased UV chroma. This change does not affect hue because it is independent of brightness.

The anatomical explanation for these differences probably involves changes in the thickness of the cortex and subsequent exposure of the 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 this study, we found that bacteria tended to degrade the cortex and expose the spongy layer. The loss of light-absorbing cortex (Finger 1995) may cause overall reflectance to increase through greater exposure of light to the spongy layer (Shawkey et al. 2005a). This increase could cause greater reflection of light from the spongy layer and hence higher brightness. Furthermore, such damage to the cortex would not affect the dimensions of the spongy layer itself, and this pattern is consistent with the observed constancy of hue values of the experimental group following treatment. Why UV chroma decreased in the experimental group is less clear. Reflection at very short UV wavelengths (300–320 nm) appears not to be affected by the experimental treatment (fig. 2), suggesting either that the elements of barb morphology responsible for reflection at these wavelengths were not damaged by the bacteria or that their reflective properties were not altered by damage. The lack of change in these wavelengths coupled with the overall increase in brightness of the experimental feathers may have caused a decrease in UV-V chroma. Weak or absent correlations between these color variables and one simple measure of barb damage suggests that other unmeasured types of damage, such as loss of barbules and depth of damage to the barb, may be critical to color change. Future studies should examine these and other damage variables in relation to color in wild populations of birds.

We tested the hypothesis that bacteria can directly change feather color in a lab experiment. This experiment provided direct evidence that keratinolytic bacteria are able to alter structural plumage coloration. 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. Indeed, Cristol et al. (2005) did not find any effect of inoculation with a single strain of keratinolytic bacteria on feathers of captive birds. Degradation of feathers may occur quite differently under natural conditions with the complex bacterial communities that appear to exist on feathers (Lucas et al. 2005; Shawkey et al. 2005a). Future studies should inoculate entire assemblages of bacteria on feathers.

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Figure 5: Scanning electron micrograph (x 500) of the outer surface of a feather barb experimentally damaged by the keratinolytic bacterium *Bacillus pumilus* and an undamaged feather barb. C = cortex, B = bacterial cells.
It is clear from our results that keratinolytic bacteria can directly alter structural plumage color, and this result is novel and potentially important for sexual signaling. A number of studies have shown evidence that females prefer males with more UV-reflective feathers in species with both noniridescent (Andersson et al. 1998; Hunt et al. 1999; Sheldon et al. 1999) and iridescent (Bennett et al. 1997) colors. Other studies have found evidence that the brightness and UV-V chroma of structural coloration signals the resource-holding potential of males (Keyser and Hill 1999; Siefferman and Hill 2005). 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. Before we confer an important role to feather-degrading bacteria in the signal content of blue feathers, we will first have to show that the type of damage shown in our experiment occurs in wild populations of birds and that it is correlated with measures of color. Bacteria may not degrade feathers so extensively in the wild and may have beneficial or commensal roles.

Perhaps most important, these data also suggest that, contrary to the dominant paradigm (reviewed in Hill and McGraw 2006), brightness may not always be a reliable signal of male quality. More damaged feathers are brighter, and birds may thus 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). Perhaps achromatic variables such as brightness that can be heavily altered by environmental damage (Ornborg et al. 2002; this study) are not as reliable as those that are not so susceptible, such as hue. Alternatively, the aerodynamic or insulation properties of damaged feathers may be superior to those of undamaged feathers. All of these hypotheses will require further research.

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. The potential for interactions between microbes and feathers to affect the signal content of structural color display is enormous and should be explored through correlative and experimental studies.

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