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GLOGER'S RULE, FEATHER-DEGRADING BACTERIA, AND COLOR VARIATION AMONG SONG SPARROWS

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Abstract. Feathers tend to be darkly colored in habitats where relative humidity is high and pale where it is low. We suggest that this correlation, known as Gloger's rule, results, in part, from selection for dark feathers that are more resistant than light feathers to bacterial degradation, which is a severe problem in humid habitats where bacteria thrive and a lesser problem in arid habitats. In May and June 2000–2002 we sampled feather-degrading bacteria (*Bacillus licheniformis*) from the plumage of Song Sparrows (*Melospiza melodia*) in southeastern Arizona and northwestern Washington. Under standardized laboratory conditions, feather-degrading bacteria from the plumage of sparrows in the humid Northwest degraded feathers more rapidly and more completely than feather-degrading bacteria from sparrows of the arid Southwest. The differences in feather-degrading bacteria and in relative humidity produce a difference in the intensity of selection, which in turn produces the difference in color described in Gloger's rule.

Key words: Bacillus licheniformis, *color, feather, melanin,* Melospiza melodia, *Song Sparrow.*

La Regla de Gloger, Bacterias Degradantes de Plumas y Variación de Color en *Melospiza melodia*

Resumen. Las plumas tienden a ser de tonos obscuros en hábitats donde la humedad relativa es alta y más pálidas en hábitats donde la humedad relativa es baja. Esta correlación, conocida como la regla de Gloger, se aplica a muchas especies de aves a través del mundo. Sugerimos que la regla de Gloger es, en parte, un producto evolutivo de la selección por plumas obscuras, que son más resistentes a la degradación bacteriana que las plumas claras. La degradación bacteriana es un problema severo en hábitats húmedos donde prosperan las bacterias y un problema menor en hábitats áridos. En mayo y junio de 2000 a 2002 tomamos muestras de bacterias degradantes de plumas (*Bacillus licheniformis*) del *Melospiza melodia fallax,* que tiene plumaje pálido y reside en la parte sureste del estado de Arizona, y comparamos la incidencia y actividad de estas bacterias con las de aquellas encontradas en el plumaje obscuro de *M. m. morphna*, que reside de los bosques húmedos del noroeste del estado de Washington. Sin embargo, bajo condiciones estandarizadas de laboratorio, las bacterias obtenidas de *M. m. morphna*, degradaron las plumas más rápida y completamente que las bacterias de *M. m. fallax*. Las diferencias sugieren que las plumas obscuras de *M. m. morphna* del noroeste húmedo están sujetas a selección más intensa para resistir la degradación bacteriana que las plumas claras del gorrión del suroeste árido. La diferencia en humedad relativa produce una diferencia en la intensidad de selección, que a su vez produce la diferencia en color descrita en la regla de Gloger.

Feather degradation by *Bacillus licheniformis* (Williams et al. 1990), its occurrence on the feathers of wild birds (Burtt and Ichida 1999, Muza et al. 2000), and the increased resistance of melanic feathers to bacterial degradation (Goldstein et al. 2004), raise the possibility that plumage color may be an evolutionary response to the presence of feather-degrading bacteria. In 1833 Gloger noted that birds in climates with high relative humidity were darker than conspecifics in climates with low relative humidity. Zink and Remsen (1986) found that among birds of the United States and Canada individuals in 94% of the species were darker in areas of high relative humidity than in areas of low relative humidity. Because bacteria thrive in humid habitats and because dark, melanic feathers resist bacterial degradation better than light feathers that lack melanin, we hypothesized that the geographic correlation of dark coloration with high relative humidity may be a response to selection for feathers that resist bacterial degradation.

Our test species was the Song Sparrow (*Melospiza melodia*), which is widely distributed and abundant (Arcese et al. 2002), easily captured, and known to harbor feather-degrading bacteria in its plumage (Burtt and Ichida 1999). We sampled individuals from a pale subspecies of the arid southwestern United States and from a dark subspecies of the humid northwestern coast. If feather-degrading bacilli have been a factor in selection for the observed color difference, then *B. licheniformis* should occur on a higher proportion of dark, northwestern Song Sparrows than on their pale southwestern relatives.

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METHODS

BACTERIAL SAMPLING OF SONG SPARROWS

We quantified color variation and sampled plumage microorganisms in two populations of Song Sparrows, pale individuals of the subspecies *M. m. fallax* of southeastern Arizona and dark individuals of the subspecies *M. m. morphna* of western Washington (Arcese et al. 2002). In Arizona we captured sparrows in mist nets set along Sonoita Creek (31°31′N, 110°46′W) near Patagonia and along the San Pedro River (31°33′N, 110°08′W) near Sierra Vista. In Washington we captured sparrows in 3–5-year-old clearcuts near Forks (48°N, 124°W) and in gardens, fields and woodland edges in Clinton (47°59′N, 122°23′W).

We disinfected our hands with quaternary disinfectant and allowed them to air dry before removing a bird from the net. Each bird's back was rubbed with a sterile, Dacron-tipped applicator (Puritan, St. Louis, Missouri) wetted with sterile saline (0.85% NaCl). After the sample was collected the applicator was replaced in its sterile envelope. The tail and venter were sampled with the same procedure. The plumage of the back, tail, and venter was then pressed lightly on three separate trypticase soy agar (TSA; Acumedia, Troy, Michigan) plates. TSA is a rich medium on which many different microorganisms grow readily. Exposed applicators, in their sterile envelopes, and exposed plates wrapped in clean plastic sandwich bags were placed in a Styrofoam cooler with ice packs, where they remained until we returned to the laboratory 4–6 hr later. After the microbial samples were collected the birds were banded with a U.S. Fish and Wildlife Service band and released. Individuals caught more than once on the same day were sampled on the initial capture only.

In the laboratory we removed the applicators from their envelopes, placed each in an individually labeled, sterile tube of modified (pH 7.5, 7.5% NaCl) nutrient broth (BBL, Cockeysville, Maryland), and incubated the tubes for 7 days at 50°C. *B. licheniformis* forms spores under adverse conditions, such as occurred on the Dacron-tipped applicators, but emerges as a vegetative cell when conditions improve, such as occurred in the broth (Black 1996).

The slightly basic, moderately saline broth and high temperature favor the growth of *B. licheniformis* and inhibit the growth of most other microorganisms (Burtt and Ichida 1999). If the broth remained clear, the sample lacked *B. licheniformis* and was discarded. If the broth became cloudy, the bacilli were cultured by streaking a loopful of the medium across a sterile TSA plate and incubating at 36°C. After 48 hr we checked the plate to be sure that it contained only *B. licheniformis*, which forms wrinkled, cone-shaped colonies. If the culture was pure, we removed a representative colony and transferred it to a TSA slant. The slant was incubated for 48 hr at 36°C, then was removed and stored at 4°C until further testing. The combination of selective media, high incubation temperature, and visual identification of isolated colonies grown from two sequential transfers of bacteria from the media has been shown to successfully isolate *B. licheniformis* (Burtt and Ichida 1999).

Field samples on TSA plates were incubated at 36°C. After 48 hr all plates were removed from the incubators and *B. licheniformis* colonies were identified and counted. We used a sterile loop to transfer colonies of *B. licheniformis* to individually labeled, sterile tubes of modified nutrient broth and processed them as described above for the applicators. The procedure screened out strains of *B. licheniformis* that were neither thermophilic nor tolerant of moderately saline and mildly alkaline conditions. Such strains do not degrade feathers (Burtt and Ichida 1999).

All isolates were labeled to indicate not only the bird and area of the bird from which they were collected, but also the applicator or plate on which they were initially cultured.

MEASURING INTRASPECIFIC COLOR VARIATION

Before release the colors of each Song Sparrow were matched by eye to color samples in Kornerup and Wanscher (1989). Matching was done under clear sky at least 1 hr after dawn and no later than 1 hr before sunset. All color matching was done by EHB, who matched the dark stripes and background color of the greater wing coverts near the ''wrist'' of the wing, which could be held open against color swatches in the handbook. Then the bird was held on its back and the brown stripes of the venter and their background color were matched to the color swatches. Each sparrow was released after its colors were matched. Fifty-three individuals of *M. m. fallax* and 98 individuals of *M. m. morphna* were measured.

Luminance is a measure of the amount of light that is reflected back to the observer from the colored patch. White reflects the most light and black the least. We assigned luminance scores to markings by recording the column of the matching color swatch in Kornerup and Wanscher (1989; white = 1, dark gray = 6). Stripes that were blacker than dark gray were assigned a score of 7. Thus the luminance of the stripe or its background varied between 1 and 7.

SOIL SAMPLES

We collected soil samples at each site. We used a sterile tablespoon to dig a sample of soil and place it in a sterile plastic bag, which was then sealed. The bags were kept on ice until returned to the laboratory where a sterile, Dacron-tipped applicator was rolled in the soil, the excess particles shaken off, and the applicator placed in an individually labeled, sterile tube of modified (pH 7.5, 7.5% NaCl) nutrient broth. We incubated tubes for 7 days at 50°C. If present, *B. licheniformis* was identified and isolated as described above.

MEASURING BACTERIAL ACTIVITY

Plumage may be darker in response to the probability of colonization by feather-degrading bacilli. Alternatively the plumage of Song Sparrows may be darker in the humid Northwest in response to bacteria that degrade feathers more rapidly or more completely than bacilli that occur in the arid Southwest. To test this possibility we inoculated test tubes of feather medium (Williams et al. 1990) with each of the *B. licheniformis* isolates from Arizona and Washington sparrows and assessed feather condition (Table 1) daily for 10 days.

TABLE 1. Qualitative scale used to estimate the condition of chicken feathers exposed to feather-degrading *Bacillus licheniformis*.

Condition of feather	Description
6 (best)	Barbs attached to rachis and one another, at most only slight, mostly distal separations
5	Numerous separations among some or all barbs, none detached from rachis, solution clear, or with some small particles suspended
4	Numerous separations among barbs, small pieces in solution, <10% detached from rachis, solution slightly cloudy
3	Barbs separated from each other, 10–49% of barbs detached wholly or partially from rachis, many large and small pieces in solution
2	50–100% of barbs detached wholly or partially from rachis, solution cloudy with pieces of barbs
1	Large pieces of barbs or rachis or both present in solution, only rachis identifiable
0 (worst)	Only minute pieces of rachis or barbs visible in solution

We used secondary remiges from single comb white leghorn chickens (*Gallus domesticus*) to test bacterial isolates for feather-degrading activity. We removed and discarded the distal 1 cm from the feather; the next 4 cm were removed, cut in half across the rachis, and the two halves placed in a test tube. We added 10 mL of feather medium (Williams et al. 1990) to each tube. All tubes were sterilized at 121°C and 7.7 kg pressure for 15 min. We removed the bacterial isolates from storage at 4°C and inoculated fresh TSA cultures with the isolate to be tested. After 24 hr a loopful of bacteria was removed and suspended in sterile saline. The turbidity of the saline-bacterial suspension was adjusted to 0.5 MacFarland standard, which corresponds to ~150 000 cells mL^{-1}. Two drops of this suspension (~0.1 mL) were placed in a test tube of medium containing the two 2-cm pieces of feather. A replicate was prepared from the same suspension. Tubes were placed in a rack in a shaker incubator at 50°C and rotated at 125 rpm.

We checked all tubes daily for degradation of the feather pieces. Racks were removed from the incubator one at a time. Each test tube was removed from the rack and held against a vortexer until a whirlpool extended from the surface of the solution to the bottom of the test tube. This ensured that all materials were thoroughly mixed and that each sample achieved the same level of mixing every day. The contents were evaluated visually using the criteria in Table 1.

All feathers were condition 6 when placed in the medium, but after sterilization in the autoclave, many of the feathers were rated condition 5. Occasionally a feather was rated condition 4 after sterilization and before inoculation with *B. licheniformis*, but none were rated lower than 4. Therefore we considered the feather to be degraded when both pieces in the test tube reached condition 3 or lower (Table 1).

STATISTICAL ANALYSES

Most of our data were not normally distributed; therefore most of our analyses were nonparametric (Minitab 2000). We used the Mann-Whitney *U*-test and its normal approximation (Zar 1999) to compare the luminance values of the stripe and background colors of the back and venter of Song Sparrows in southern Arizona to those of Song Sparrows in northwestern Washington. We used the Mann-Whitney *U*-test again to compare the condition of feathers (Table 1) exposed to different isolates of *B. licheniformis*. We used chi-square with a continuity correction (Zar 1999) to test for independence of the proportion of Song Sparrows with *B. licheniformis* in their plumage in Arizona and Washington. We also used the chi-square, again with the continuity correction, to compare the proportion of feathers stripped of barbs by bacilli isolated from Song Sparrows in Arizona and Washington. We used the *t*-test to compare the number of days required to degrade feathers by *B. licheniformis* isolated from Song Sparrows in Arizona and Washington. Luminance and feather condition values are given as modes. Days to degrade are given as means ± SE.

RESULTS

INTRASPECIFIC COLOR VARIATION

The luminance values of the stripes and background colors of Song Sparrows clustered tightly about modal values characteristic of that marking for that subspecies. The venter of *M. m. fallax* was white (luminance 1) in all 53 sparrows measured, whereas the venter of *M. m. morphna* was significantly grayer (luminance 2, range 1–5; $U = 2406$, $P < 0.001$; Fig. 1). The breast stripes were dark in *M. m. fallax* (luminance 5, range 4–6), but significantly darker in *M. m. morphna* (luminance 6, range 5–6; $U = 2634$, $P < 0.001$; Fig. 1). The dorsal background color as measured on the greater wing coverts was brown (luminance 5, range 2–5) in *M. m. fallax*, but significantly darker (luminance 6, range 5–6; $U = 1578$, $P < 0.001$; Fig. 1) in *M. m. morphna*. Finally, the dorsal stripes of *M. m. fallax* had a modal luminance of 6 (range 5–6), but those of *M. m. morphna* were still darker, almost black (luminance 7, range 6–7; $U = 1762$, $P < 0.001$; Fig. 1).

SOIL SAMPLES

Soils at all of our sites contained feather-degrading *B. licheniformis*. The bacilli were active, vegetative cells under humid to damp conditions (Ichida et al. 2001),

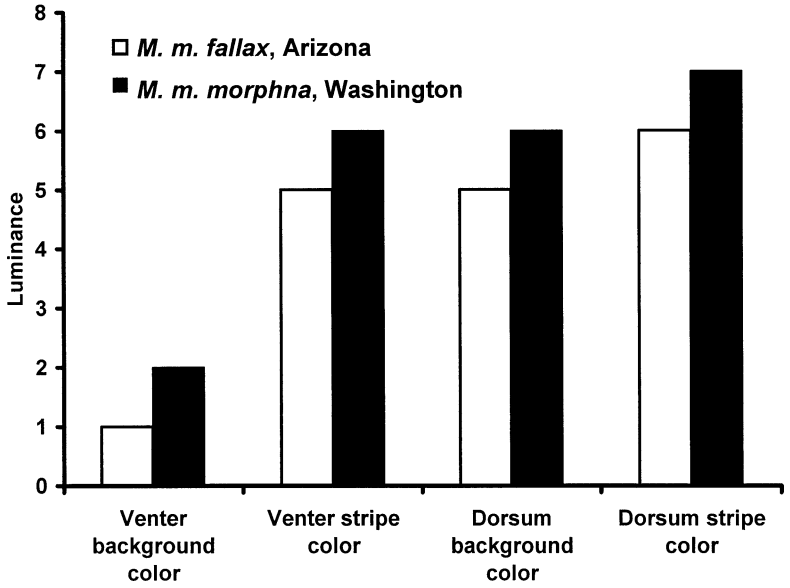

FIGURE 1. Comparison of the modal luminance of the dorsal and ventral stripes and backgrounds of Song Sparrows in arid Arizona (*Melospiza melodia fallax*; $n = 53$) and humid Washington (*M. m. morphna*; $n = 98$). Luminance ranges from 1 (white) to 7 (almost black). For each marking the difference between the subspecies was significant at $P < 0.001$, with *fallax* always lighter than *morphna*.

and formed inactive endospores under arid conditions (Black 1996).

OCCURRENCE OF FEATHER-DEGRADING BACILLI IN SONG SPARROWS

Feather-degrading bacilli occurred on 33 of 142 (23%) Song Sparrows (*M. m. morphna*) from the humid Northwest and 14 of 77 (18%) from the arid Southwest (*M. m. fallax*), a nonsignificant difference ($\chi^2_1 = 0.6$, $P = 0.48$). Despite the small difference, individuals of *M. m. morphna* were more likely to have feather-degrading bacilli in their plumage than individuals of *M. m. fallax* in all 3 years studied. The difference may be small, but our limited data suggest that it may be consistent.

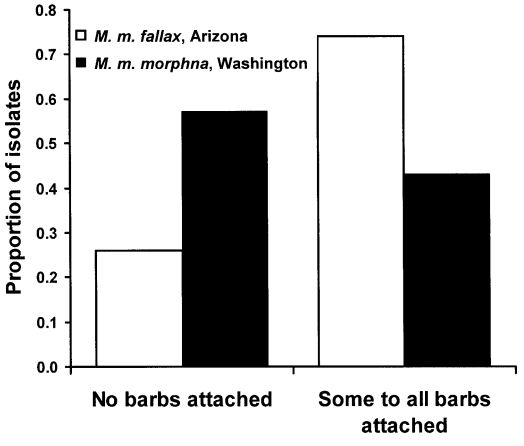

FIGURE 2. Proportion of bacilli isolated from the plumage of Song Sparrows in arid Arizona (*M. m. fallax*; $n = 18$) and humid Washington (*M. m. morphna*; $n = 47$) that stripped all barbs from the rachis of chicken feathers within 10 days of inoculation.

BACTERIAL ACTIVITY

Among white chicken feathers that degraded, those exposed to bacilli from the plumage of Washington Song Sparrows degraded more rapidly (5.1 ± 0.4 days) than those exposed to bacilli from the plumage of Arizona Song Sparrows (6.8 ± 0.6 days; $t_{31} = 2.5$, $P = 0.02$). At the end of the 10-day experiment, the modal condition of feathers degraded by bacilli from dark sparrows was 1 (range 0–3), whereas the modal condition of feathers degraded by bacilli from pale sparrows was 2 (range 0–3). The difference was significant ($U = 742$, $P = 0.02$). Significantly more bacilli from dark sparrows ($\chi^2_1 = 5.2$, $P = 0.03$; Fig. 2) reduced white chicken feathers to condition 1 or 2 (Table 1) than bacilli isolated from pale sparrows.

DISCUSSION

We confirmed quantitatively that all markings of Song Sparrows from the humid coastal forests of northwestern Washington are darker than the comparable markings of Song Sparrows from the arid Southwest, a pattern described previously by Zink and Remsen (1986), Aldrich and James (1991), James (1991), and Arcese et al. (2002). We found feather-degrading *B. licheniformis* in soils and on Song Sparrows of both regions, but feather-degrading bacilli isolated from the plumage of dark Song Sparrows degraded feathers

more quickly and more completely than feather-degrading bacilli from the plumage of pale Song Sparrows. This result suggests that feathers of Song Sparrows in the humid Northwest are subjected to strong selection pressure to develop resistance to bacterial feather degradation.

The darker color indicates more melanin in the feathers of *morphna*, which inhabits the humid forests of the Northwest coast, than in the feathers of *fallax*, which inhabits the arid Southwest. The presence of melanin increases the abrasion resistance and thickness of feather keratin (Burtt 1979, 1986, Voitkevich 1966). The inclusion of granular melanin also increases the hardness of keratin (Bonser 1995, Bonser and Witter 1993), which increases its resistance to abrasion, independent of thickness. Recent evidence (Goldstein et al. 2004) indicates that melanin also increases the resistance of feather keratin to bacterial degradation. Regardless of whether the increased resistance is due to thicker or harder keratin, we would expect Song Sparrows in a climate with high relative humidity to evolve melanic plumage as a defense against bacterial degradation of their feathers. Thus selection would favor dark plumage in environments with high relative humidity, which is the pattern observed by Gloger (1833). That this is a dynamic, evolutionary relationship was shown by Johnston and Selander (1971), who documented the darkening of House Sparrows (*Passer domesticus*) as they spread into regions of the United States and Canada with high relative humidity during the last 150 years.

The plumage of birds hosts an entire ecosystem of microorganisms, among which are other feather-degrading species, for example the bacterium *Streptomyces pactum* (Bockle et al. 1995) and the fungi *Chrysosporium* spp. (Pugh and Evans 1970) and *Fusarium sporotrichioides* (H. M. Costello, pers. comm.). The combined action of several feather-degrading species may have a greater effect on feathers than we have demonstrated by focusing on *B. licheniformis*. However, the combined action may be subtle due to competition among microorganisms that are able to chemically inhibit or kill each other (Burtt 1999). Additionally, the bird may be able to limit feather degradion directly by exposing feathers to ultraviolet irradiance or heat (Moyer and Wagenbach 1995) during sunning, or indirectly by preening, dust bathing, or other maintenance behavior that could encourage the growth of microbes that excrete antibacterial chemicals (e.g., *Pencillium* spp.).

Gloger's rule describes color variation in 94% of the North American species that encounter substantial climatological differences in relative humidity (Zink and Remsen 1986). This is a far more robust relationship than that described by Bergmann's or Allen's rules (Zink and Remsen 1986). We suggest that the strength of the relationship may be the product of several important selection pressures (Table 2), all of which favor dark color in a humid environment. None of the pressures listed in Table 2 are mutually exclusive, and resistance to bacterial degradation is simply another pressure that selects for dark feathers. We believe the strength of the relationship between relative humidity and color rests on the number of selection pressures that act in concert to favor the evolution of dark plumage in habitats with high relative humidity.

TABLE 2. Selection pressures that may contribute to the evolution of dark coloration among species of birds living in climates with high relative humidity.

Selection pressure	Source
Background matching	Zink and Remsen 1986
Enhanced drying	Burtt 1981
Environmental plasticity	James 1991
Physiological constraints	Buxton 1923
Resistance to bacterial degradation	This study
Thermoregulation	Walsberg 1983

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LITERATURE CITED

ALDRICH, J. W., AND F. C. JAMES. 1991. Ecogeographic variation in the American Robin. Auk 108:230–249.

ARCESE, P., M. P. SOGGE, A. B. MARR, AND M. A. PATTEN. 2002. Song Sparrow (*Melospiza melodia*). *In* A. Poole and F. Gill [EDS.], The birds of North America, No. 704. The Birds of North America, Inc., Philadelphia, PA.

BLACK, J. G. 1996. Microbiology principles and applications. Prentice Hall, Upper Saddle River, NJ.

BOCKLE, B., B. GALUNSKY, AND R. MULLER. 1995. Characterization of keratolytic serine protease from *Streptomyces pactum* DSM 40530. Applied and Environmental Microbiology 61: 3705–3710.

BONSER, R. H. C. 1995. Melanin and the abrasion resistance of feathers. Condor 97:590–591.

BONSER, R. H. C., AND M. S. WITTER. 1993. Indentation hardness of the bill keratin of the European Starling, *Sturnus vulgaris*. Condor 95:736–738.

BURTT, E. H., JR. 1979. Tips on wings and other things, p. 70–123. *In* E. H. Burtt Jr. [ED.], The behavioral significance of color. Garland STPM Press, New York.

BURTT, E. H., JR. 1981. The adaptiveness of colors. BioScience 31:723–729.

BURTT, E. H., JR. 1986. An analysis of physical, physiological and optical aspects of avian coloration with emphasis on wood-warblers. Ornithological Monograph 38.

BURTT, E. H., JR. 1999. Think small. Auk 116:872–874.

BURTT, E. H., JR., AND J. M. ICHIDA. 1999. Occurrence of feather-degrading bacilli in the plumage of birds. Auk 116:364–372.

BUXTON, P. A. 1923. Animal life in deserts. Edward Arnold and Co., London.

GLOGER, C. L. 1833. Das Abändern der Vögel durch Einfluss des Klimas. Breslau, Germany.

GOLDSTEIN, G., K. FLORY, B. A. BROWNE, S. MAJID, J. M. ICHIDA, AND E. H. BURTT JR. 2004. Bacterial degradation of black and white feathers. Auk, in press.

JAMES, F. C. 1991. Complementary descriptive and experimental studies of clinal variation in birds. American Zoologist 31:694–706.

JOHNSTON, R. F., AND R. K. SELANDER. 1971. Evolution in the House Sparrow. II. Adaptive differentiation in North American populations. Evolution 25:1–28.

KORNERUP, A., AND J. H. WANSCHER. 1989. Methuen handbook of colour. 3rd ed. Methuen, London.

MINITAB. 2000. Statistics, release 13.20 for Windows. MINITAB Inc., State College, PA.

MOYER, B. R., AND G. E. WAGENBACH. 1995. Sunning by Black Noddies (*Anous minutus*) may kill chewing lice (*Quadraceps hopkinsi*). Auk 112:1073–1077.

MUZA, M. M., E. H. BURTT JR., AND J. M. ICHIDA. 2000. Distribution of bacteria on feathers of some eastern North American birds. Wilson Bulletin 112:432–435.

PUGH, G. J. F., AND M. D. EVANS. 1970. Keratinophilic fungi associated with birds. Transactions of the British Mycological Society 54:241–250.

VOITKEVICH, A. A. 1966. The feathers and plumage of birds. Sidgwick and Jackson, London.

WALSBERG, G. E. 1983. Avian ecological energetics. Avian Biology 7:161–220.

WILLIAMS, C. M., C. S. RICHTER, J. M. MACKENZIE JR., AND J. C. H. SHIH. 1990. Isolation, identification, and characterization of a feather-degrading bacterium. Applied and Environmental Microbiology 56:1509–1515.

ZAR, J. H. 1999. Biostatistical analysis. 4th ed. Prentice-Hall, Englewood Cliffs, NJ.

ZINK, R. M., AND J. V. REMSEN JR. 1986. Evolutionary processes and patterns of geographic variation in birds. Current Ornithology 4:1–69.

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MELANIN BASIS OF ORNAMENTAL FEATHER COLORS IN MALE ZEBRA FINCHES

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Abstract. The carotenoid-pigmented bill of Zebra Finches (*Taeniopygia guttata*) has received much recent attention as a sexually selected signal of quality, but these birds also display several sexually dichromatic plumage traits, including rust-colored cheek patches, a black breast band, and brown flanks. Black, brown, and earth-toned features in animals are thought to be produced by melanin pigments, but few studies have identified the melanin content of such colors in bird feathers. We used a series of biochemical techniques to investigate the pigmentary basis of these plumage colors in male Zebra Finches. All three feather traits contained melanin pigments, but varied in the amounts of the two basic forms of melanin (eumelanin and phaeomelanin). Black breast feathers contained predominantly eumelanin, whereas cheek and flank feathers contained extraordinarily high concentrations of phaeomelanin. Conventional methods of carotenoid analysis detected no carotenoids in either the cheek or flank feathers.

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Coloración Basada en Melaninas en las Plumas Ornamentales de los Machos de *Taeniopygia guttata*

Resumen. El pico pigmentado con carotenoides de *Taeniopygia guttata* ha sido destacado recientemente como una señal de calidad seleccionada sexualmente, pero estas aves también presentan varios caracteres de plumaje sexualmente dicromáticos, incluyendo parches en las mejillas de color óxido, una faja pectoral negra y flancos de color café. Se cree que las tonalidades negras, cafés y color tierra son producidas por melaninas en los animales, pero existen pocos estudios que hayan identificado el contenido de melanina de dichos colores en las plumas de las aves. En este estudio empleamos una serie de técnicas bioquímicas para investigar la base pigmentaria de estos colores del plumaje en machos de *T. guttata*. Los tres caracteres de las plumas contaron con pigmentos melánicos, pero variaron en las cantidades de las dos formas básicas de melanina (eumelanina y feomelanina). Las plumas negras del pecho presentaron principalmente eumelanina, mientras que las de las mejillas y los flancos presentaron concentraciones extraordinariamente altas de feomelanina. Los métodos tradicionales de análisis de carotenoides no detectaron este tipo de pigmentos en las plumas de las mejillas y los flancos.

Color ornaments are some of the best-studied sexually selected traits in animals, and several recent reviews have emphasized the signaling function of ornamental colors in bird feathers (Olson and Owens 1998, Hill 1999, Senar 1999, Møller et al. 2000, Jawor and Breitwisch 2003). Birds produce colors in two ways, using pigments or microstructure. Pigments come in a variety of forms, but the two main classes found in ornamental bird feathers are carotenoids (red, orange, and yellow) and melanins (brown and black; Fox and Vevers 1960). An emerging thrust of this research on avian sexual colors is that we can gain important insights about the information content and functional significance of plumage colors by understanding the proximate mechanisms that control their expression (McGraw and Hill 2000). Thus, we must first characterize the types of pigments that confer color on feather patches and later investigate how certain environmental, physiological, and genetic factors mediate color production.

Biochemical techniques that identify carotenoid pigments in bird feathers have existed for decades (reviewed in Hudon and Brush 1992). Recently, they have been improved through advances in chromatographic procedures (e.g., high-performance liquid chromatography, or HPLC; Stradi et al. 1995). Methods for studying melanins in bird feathers, however, have lagged behind. Only within the last few decades have biochemical procedures been employed to characterize melanins in animal tissues (Ito and Fujita 1985). Two general classes of melanin pigments exist: the yellow or rufous phaeomelanins and the black, gray, or brown eumelanins (Prota 1992). These classes exist in varying proportions in the three species in which the melanin content of colorful plumage has been investigated: domestic pigeons (*Columba livia*; Haase et al. 1992), Mallards (*Anas platyrhynchos*; Haase et al. 1995), and Japanese Quail (*Coturnix japonica*; Shiojiri et al. 1999).

We studied the pigmentary basis of colorful plumage ornaments in the Zebra Finch (*Taeniopygia guttata*). This Australian passerine has been the subject of several recent tests of sexual-selection and honest-signaling theory, specifically focused on the carotenoid-based coloration of the beak in males (Blount, Metcalfe, Birkhead, and Surai 2003, Blount, Metcalfe, Arnold et al. 2003, McGraw and Ardia 2003, McGraw et al. 2003). Male finches also display a series of sexually dichromatic plumage features, however; they exhibit patches of rust-colored cheek feathers, black breast feathers, and brown flank feathers that are all absent in females. We presumed that all feather patches were colored by melanins, and followed previously published biochemical procedures (Haase et al. 1992) to assay both phaeomelanin and eumelanin content in these feathers. We also performed conventional methods of carotenoid analysis (Hudon and Brush 1992, McGraw, Hill et al. 2002) to determine whether carotenoid pigments were present in rusty or brown feathers.

METHODS

FEATHER COLLECTION

We plucked feathers from six male Zebra Finches in wild-type plumage. The birds came from two captive populations: one at Cornell University ($n = 3$; McGraw, Adkins-Regan, and Parker 2002) and one at the University of California-Davis ($n = 3$; Millam et al. 2001). Because cheek feathers are much smaller than breast and flank feathers, we plucked 20 rust-colored cheek feathers, 8 black breast feathers, and 8 brown flank feathers from each bird. Feathers from each region were split into two equal portions and were immediately placed into 1.5-mL Eppendorf tubes and stored in an envelope at room temperature until feathers were analyzed biochemically.

CAROTENOID ANALYSES

We trimmed 3–5 mg of rusty and brown barbules from feathers, washed them sequentially in ethanol and hexane (solvent washes yielded no carotenoids), and blotted them dry. We added 1 mL acidified pyridine to the barbules in a 9-mL glass tube, filled the headspace of the tube with argon (to prevent pigment oxidation), and held the tube at 95°C for 4 hr. After this time, we cooled the tube to room temperature, added 1 mL water, inverted the tube a few times, and then added 3 mL hexane:*tert*-butyl methyl ether (1:1, v:v; to recover both polar and nonpolar lipids). We shook the tube vigorously for 2 min, centrifuged for 5 min at 3000 RPM, transferred the supernatant to a fresh tube, and evaporated the solvent to dryness under a stream of nitrogen. We ran a positive control (yellow, carotenoid-pigmented contour feathers from American Goldfinches [*Carduelis tristis*]; McGraw et al. 2001) along with our samples.

Residues were redissolved in 200 μL HPLC mobile phase, and 50 μL was injected into a Waters™ 717plus Autosampler HPLC (Millipore Corp., Bedford, Massachusetts) fitted with a Develosil RPAqueous RP-30 column (250 × 4.6 mm; Nomura Chemical Co. Ltd., Aichi, Japan) and a column heater (Eppendorf TC-50, Hamburg, Germany) set at 27°C. We used two different isocratic systems (Hewlett-Packard 1050 Series Isocratic Pump), both at a constant flow rate of 1.2 mL per min, to analyze xanthophylls and carotenes separately, if they were present. For xanthophylls, we used acetonitrile:methanol:chloroform (46:46:48, v:v:v) as the mobile phase. For carotenes, we used methanol: dichloromethane (50:50, v:v) as the mobile phase. Data were collected from 250–600 nm using a Waters™ 996 photodiode array detector (Waters Corporation, Milford, Massachusetts). The minimum detection limit of our PDA detector was 0.0001 AU (absorbance units), which amounts to approximately 1 ng of carotenoid per 50 μL injection or 0.005 mg of carotenoid per gram of pigmented feather portion.

MELANIN ANALYSES

To determine eumelanin concentration, 3–5 mg of rusty, black, or brown barbules were trimmed and homogenized in water (1:100, w:v). 400 μL of the feather–water homogenate were added to 800 μL 1M H_2SO_4, oxidized with 3% $KMnO_4$. The resulting oxidation product (pyrrole-2,3,5-tricarboxylic acid; PTCA) was analyzed via HPLC (Ito and Fujita 1985, Ito and Wakamatsu 1994). Phaeomelanins were examined by hydrolyzing 200 μL feather homogenate with 500 μL 57% hydriodic acid at 130°C in the presence of H_3PO_2 for 24 hr, and subsequently analyzing the product (4-amino-3-hydroxyphenyalanine; 4-AHP) using HPLC with electrochemical detection (Wakamatsu et al. 2002). Amounts (mg per g of feather) of eumelanin and phaeomelanin were obtained by multiplying the amount of PTCA and 4-AHP by conversion factors of 50 and 9, respectively (Ito and Fujita 1985, Wakamatsu and Ito 2002).

RESULTS

CAROTENOID ANALYSES

Using our thermochemical extraction procedure, we found that none of the orange or brown feathers in male Zebra Finches contained any carotenoid pigments. Similar analyses using a more mild, mechanical extraction method also failed to detect any carotenoids in cheek feathers (R. Stradi, unpubl. data).

MELANIN ANALYSES

All three feather types analyzed contained high concentrations of melanin pigments. As expected for a black color, breast feathers contained predominantly (92%) eumelanin (mean ± SE = 13.6 ± 2.8 mg eumelanin per g feather and 1.1 ± 0.3 mg phaeomelanin per g feather; Fig. 1). Cheek and flank patches, in contrast, contained predominantly phaeomelanin (99.2% and 92.3% of total, respectively) and at extraordinarily high concentrations (Fig. 1). Cheek feathers contained an average of 46.8 ± 8.3 mg phaeomelanin per g feather and 0.3 ± 0.05 mg eumelanin per g feather, whereas flank patches contained even higher concentrations (55.3 ± 10.6 mg phaeomelanin per g feather, 3.8 ± 1.2 mg eumelanin per g feather). Cheek feathers thus appear more orange and less brown than flank feathers because of their lower overall melanin concentration and a slightly higher relative amount of phaeomelanin.

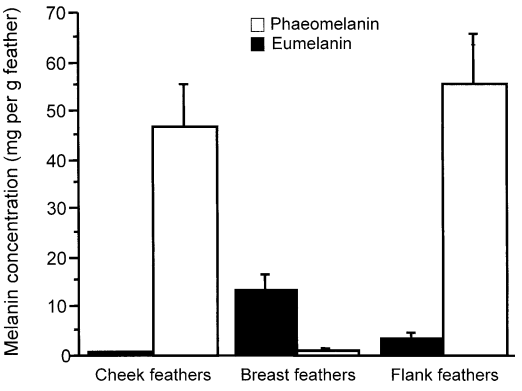

FIGURE 1. Mean (+ SE) concentrations of phaeomelanins and eumelanins found in feathers from male Zebra Finches: cheek (rusty), breast (black), and flank (brown).

DISCUSSION

Chestnut and rufous feathers reportedly harbor carotenoid pigments in certain species (e.g., Barn Swallows [*Hirundo rustica*]; Stradi 1998, Saino et al. 1999), but we found no evidence that feathers of a similar color in male Zebra Finches contained any carotenoids. Instead, melanins bestow the rust, brown, (and black) colors on these feathers.

As anticipated, the black breast patch of males was mostly eumelanic and exhibited a similar concentration (ca. 15 mg total melanins per g feather) and eumelanin:phaeomelanin ratio (92:8) to black feather pigments in Japanese Quail (Shiojiri et al. 1999). However, the cheek and flank feathers were quite unusual, having unparalleled concentrations of total melanins and high proportions of phaeomelanin. Even the richest reddish-brown feathers in pigeons contain just over 40 mg melanin per g of feather and no more than 93% of this is phaeomelanin (Haase et al. 1992). For comparison, red hair in humans also contains ca. 94% phaeomelanin, but a total melanin concentration of only 3.5 mg per g (Ito and Fujita 1985). In fact, the melanin profile of cheek feathers in Zebra Finches is most similar to the yellow hair of a certain strain of lab mice (i.e., C57BL, A^y/a), which is 99.1% phaeomelanin but still contains only ca. 26.5 mg melanin per g of hair (Ito and Wakamatsu 2003).

Melanins may occur at such high concentrations in Zebra Finch feathers due to the large amounts needed to confer rich ornamental colors. Carotenoids that color bird feathers, for example, have extremely high extinction coefficients (a measure of the light-reflecting properties of molecules; Bauernfeind 1981) and occur in the sexually selected plumage of male American Goldfinches and House Finches (*Carpodacus mexicanus*) at concentrations of no more than 2 mg per g

(Inouye et al. 2001, McGraw, Hill et al. 2002). In contrast, the extinction coefficients for phaeomelanins and eumelanins are more than an order of magnitude lower than carotenoids (Sarna and Swartz 1988), which means that more pigment is required to generate an equally rich color.

The differences in phaeomelanin and eumelanin concentrations in Zebra Finch feather areas underscore the remarkably flexible and selective capacities that animals have to synthesize and incorporate these melanins in tissues. Some mammals (e.g., mice, Ito and Fujita 1985; cats, Anderson et al. 2002) and birds (e.g., quail, Shiojiri et al. 1999) even produce individual hairs or feathers that are striped or barred, with different levels of phaeomelanin and eumelanin in each region. These studies and ours suggest that feather follicles (like hair follicles) exert very fine, local control over the rates of melanin deposition into different tissues.

The reasons why the two types of melanin appear in different amounts in bird feathers, however, are poorly understood. Synthetic pathways toward phaeo- and eumelanogenesis are thought to differ, most notably in the role of the amino acid cysteine, which acts in phaeomelanin synthesis only (Land and Riley 2000). Amino-acid availability in the diet affects melanin distribution and coat color in cats (Anderson et al. 2002), but no comparable study is yet available in birds. We do know that factors like circulating sex-hormone levels in Mallards affect feather-melanin composition, where phaeomelanin content in certain body regions is elevated in birds having higher testosterone titers (Haase et al. 1995). Whether local hormone production or circulation can explain why Zebra Finch breast feathers become primarily eumelanic and flank and cheek feathers phaeomelanic should be an interesting topic to pursue.

Likewise, the social and sexual significance of these melanic ornamental colors in Zebra Finches require additional study. Female Zebra Finches are known to preferentially mate with males having the most symmetrical patch of black breast feathers (Swaddle and Cuthill 1994), but no studies have sought to identify a signaling function of the cheek or flank ornaments. Since different types of pigmentary and structural colors often communicate different sets of information about the overall quality of males (McGraw and Hill 2000, Senar et al. 2003), Zebra Finches should serve as an ideal model to test whether different types of melanin-based features serve equally different roles in visual communication.

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LITERATURE CITED

ANDERSON, P. J. B., Q. R. ROGERS, AND J. G. MORRIS. 2002. Cats require more dietary phenylalanine or tyrosine for melanin deposition in hair than for maximal growth. Journal of Nutrition 132:2037–2042.

BAUERNFEIND, J. C. 1981. Carotenoids as colorants and vitamin A precursors: technological and nutritional applications. Academic Press, New York.

BLOUNT, J. D., N. B. METCALFE, K. E. ARNOLD, P. F. SURAI, G. L. DEVEVEY, AND P. MONAGHAN. 2003. Neonatal nutrition, adult antioxidant defences and sexual attractiveness in the Zebra Finch. Proceedings of the Royal Society of London Series B 270: 1691–1696.

BLOUNT, J. D., N. B. METCALFE, T. R. BIRKHEAD, AND P. F. SURAI. 2003. Carotenoid modulation of immune function and sexual attractiveness in Zebra Finches. Science 300:125–127.

FOX, H. M., AND G. VEVERS. 1960. The nature of animal colors. Macmillan, New York.

HAASE, E., S. ITO, A. SELL, AND K. WAKAMATSU. 1992. Melanin concentrations in feathers from wild and domestic pigeons. Journal of Heredity 83:64–67.

HAASE, E., S. ITO, AND K. WAKAMATSU. 1995. Influences of sex, castration, and androgens on the eumelanin and phaeomelanin contents of different feathers in wild Mallards. Pigment Cell Research 8:164–170.

HILL, G. E. 1999. Mate choice, male quality, and carotenoid-based plumage coloration. Proceedings of the International Ornithological Congress 22: 1654–1668.

HUDON, J., AND A. H. BRUSH. 1992. Identification of carotenoid pigments in birds. Methods in Enzymology 213:312–321.

INOUYE, C. Y., G. E. HILL, R. MONTGOMERIE, AND R. D. STRADI. 2001. Carotenoid pigments in male House Finch plumage in relation to age, subspecies, and ornamental coloration. Auk 118:900–915.

ITO, S., AND K. FUJITA. 1985. Microanalysis of eumelanin and phaeomelanin in hair and melanomas by chemical degradation and liquid chromatography. Analytical Biochemistry 144:527–536.

ITO, S., AND K. WAKAMATSU. 1994. An improved modification of permanganate oxidation that gives constant yield of pyrrole-2,3,5-tricarboxylic acid. Pigment Cell Research 1:141–144.

ITO, S., AND K. WAKAMATSU. 2003. Quantitative analysis of eumelanin and phaeomelanin in humans, mice, and other animals: a comparative review. Pigment Cell Research 16:523–531.

JAWOR, J. M., AND R. BREITWISCH. 2003. Melanin ornaments, honesty, and sexual selection. Auk 120: 249–265.

LAND, E. J., AND P. A. RILEY. 2000. Spontaneous redox reactions of dopaquinone and the balance between the eumelanic and phaeomelanic pathways. Pigment Cell Research 13:273–277.

MCGRAW, K. J., E. ADKINS-REGAN, AND R. S. PARKER. 2002. Anhydrolutein in the Zebra Finch: a new, metabolically derived carotenoid in birds. Comparative Biochemistry and Physiology B 132:813–820.

MCGRAW, K. J., AND D. R. ARDIA. 2003. Carotenoids, immunocompetence, and the information content

of sexual colors: an experimental test. American Naturalist 162:704–712.

McGraw, K. J., A. J. Gregory, R. S. Parker, and E. Adkins-Regan. 2003. Diet, plasma carotenoids, and sexual coloration in the Zebra Finch (*Taeniopygia guttata*). Auk 120:400–410.

McGraw, K. J., and G. E. Hill. 2000. Differential effects of endoparasitism on the expression of carotenoid- and melanin-based ornamental coloration. Proceedings of the Royal Society of London Series B 267:1525–1531.

McGraw, K. J., G. E. Hill, R. Stradi, and R. S. Parker. 2001. The influence of carotenoid acquisition and utilization on the maintenance of species-typical plumage pigmentation in male American Goldfinches (*Carduelis tristis*) and Northern Cardinals (*Cardinalis cardinalis*). Physiological and Biochemical Zoology 74:843–852.

McGraw, K. J., G. E. Hill, R. Stradi, and R. S. Parker. 2002. The effect of dietary carotenoid access on sexual dichromatism and plumage pigment composition in the American Goldfinch. Comparative Biochemistry and Physiology B 131: 261–269.

Millam, J. R., C. B. Craig-Veit, A. E. Quaglino, A. L. Erichsen, T. R. Famula, and D. M. Fry. 2001. Posthatch oral estrogen exposure impairs adult reproductive performance of Zebra Finch in a sex-specific manner. Hormones and Behavior 40:542–549.

Møller, A. P., C. Biard, J. D. Blount, D. C. Houston, P. Ninni, N. Saino, and P. F. Surai. 2000. Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence, or detoxification ability? Avian and Poultry Biology Reviews 11:137–159.

Olson, V. A., and I. P. F. Owens. 1998. Costly sexual signals: are carotenoids rare, risky or required? Trends in Ecology & Evolution 13:510–514.

Prota, G. 1992. Melanins and melanogenesis. Academic Press, San Diego, CA.

Saino, N., R. Stradi, P. Ninni, E. Pini, and A. P. Møller. 1999. Carotenoid plasma concentration, immune profile, and plumage ornamentation of male Barn Swallows (*Hirundo rustica*). American Naturalist 154:441–448.

Sarna, T., and H. M. Swartz. 1988. The physical properties of melanins., p. 333–358. *In* J. J. Nordlund, R. E. Boissy, V. J. Hearing, R. A. King, and J. P. Ortonne [eds.], The pigmentary system: physiology and pathophysiology. Oxford University Press, New York.

Senar, J. C. 1999. Plumage coloration as a signal of social status. Proceedings of the International Ornithological Congress 22:1669–1686.

Senar, J. C., J. Figuerola, and J. Domenech. 2003. Plumage coloration and nutritional condition in the Great Tit *Parus major*: the roles of carotenoids and melanins differ. Naturwissenschaften 90:234–237.

Shiojiri, N., T. Niwa, K. Wakamatsu, S. Ito, and A. Nakamura. 1999. Chemical analysis of melanin pigments in feather germs of Japanese Quail Bh (black at hatch) mutants. Pigment Cell Research 12:259–265.

Stradi, R. 1998. The colour of flight: carotenoids in bird plumage. Solei Gruppo Editoriale Informatico, Milan, Italy.

Stradi, R., G. Celentano, and D. Nava. 1995. Separation and identification of carotenoids in bird's plumage by high-performance liquid chromatography—diode-array detection. Journal of Chromatography B 670:337–348.

Swaddle, J. P., and I. C. Cuthill. 1994. Female Zebra Finches prefer males with symmetric chest plumage. Proceedings of the Royal Society of London Series B 258:267–271.

Wakamatsu, K., and S. Ito. 2002. Advanced chemical methods in melanin determination. Pigment Cell Research 15:174–183.

Wakamatsu, K., S. Ito, and J. L. Rees. 2002. The usefulness of 4-amino-3-hydroxyphenylalanine as a specific marker of phaeomelanin. Pigment Cell Research 15:225–232.