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Increased plumage darkness of female Shiny Cowbirds *Molothrus bonariensis* in the subtropics: an adaptation to bacterial degradation?

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The Shiny Cowbird *Molothrus bonariensis* is a sexually dichromatic species, in which males have blackish-blue iridescence and females are dull brown. However, in some subtropical parts of its distribution, females show a plumage polymorphism that ranges from dull brown to dark brown and even black. Plumage melanization has been shown to protect feathers from bacterial degradation, decreasing the effects of harmful bacterial activity and thus plumage damage. In this study, we assessed whether bacterial feather-degrading activity is acting as the selective force to increase darkness in the plumage of the female Shiny Cowbirds in Argentina. We compared the degradation of female Shiny Cowbird feathers belonging to different colour morphs when exposed to bacterial strains isolated from subtropical and temperate zones of its distribution, as well as to *Bacillus licheniformis*. We did not find differences in susceptibility to bacterial degradation between brown feathers and darker feathers. These results suggest that female plumage polymorphism in Shiny Cowbirds has not arisen as a defence against bacterial feather-degrading activity.

Keywords: *Bacillus licheniformis*, bacteria, feather degradation, melanization, *Molothrus bonariensis*.

Melanization of feathers appears to be beneficial for birds in many aspects. It has been shown that increased melanization hardens the feathers (Bonser 1995, Butler & Johnson 2004), making them more resistant to abrasion (Averill 1923, Burtt 1981, 1986). Darker plumage might also be advantageous for thermoregulation (Walsberg 1983) and background matching (Zink & Remsen 1986). In addition, recent studies have revealed that melanins protect the feathers from bacterial degradation (Goldstein *et al.* 2004, Gunderson *et al.* 2008, but see Grande *et al.* 2004).

Degradation of feathers by bacilli has attracted increasing attention from researchers (Burtt 2009), especially with the isolation of the feather-degrading bacterium *Bacillus licheniformis* (Williams *et al.* 1990). The adverse effects of bacterial activity on the plumage might have favoured different antibacterial defences in birds apart from plumage melanization (Gunderson 2008). For example, moult might have evolved as a response to feather-degrading bacteria, favouring both the replacement of damaged feathers and the reduction of the bacterial load in the bird's body (Burtt & Ichida 1999). Additionally, behaviour such as preening, dusting and sunning (Clayton 1999, Lucas *et al.* 2005, Saranathan & Burtt 2007) might decrease the bacterial feather-degrading activity on feathers, whereas the preen waxes secreted from the uropygial gland have been shown to inhibit the growth of

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harmful bacteria (Shawkey *et al.* 2003, Reneerkens *et al.* 2008).

Keratinolytic activity of bacteria on feathers causes structural damage to the barbs and may impose thermoregulatory costs as a result of reduced insulation and increased heat loss, which in turn could reduce body mass and survival (Booth *et al.* 1993, Clayton 1999). In addition, degradation of the flight feathers could reduce the aerodynamic efficiency of the bird (Barbosa *et al.* 2002). Thus, adaptations that minimize the effect of bacterial degradation on a bird's plumage should be favoured by natural selection.

Although the occurrence of feather-degrading bacilli in the plumage of birds appears to be widespread (Burtt & Ichida 1999, Whitaker *et al.* 2005) and involves a diversity of taxa (Lucas *et al.* 2003, Shawkey *et al.* 2005, 2006a, Bisson *et al.* 2007), bacterial activity might differ among locations, and thus the intensity of selection might vary for several reasons. First, conditions may be more favourable for bacilli in warm and humid habitats (Shawkey & Hill 2004). Secondly, feather-degrading bacterial strains from different locations may have different degrading activity (Burtt & Ichida 2004). Thirdly, differences in a bird's foraging activity or other behavioural or ecological aspects might vary the exposure to feather-degrading soil bacteria, leading to unequal bacterial loads among individuals (Burtt & Ichida 1999, Peele *et al.* 2009).

The effect of melanins on feather degradation by bacteria has been previously studied by comparing dark feathers with feathers having no other pigments, or with feathers with carotenoids (Goldstein *et al.* 2004, Grande *et al.* 2004, Gunderson *et al.* 2008), but how melanized feathers of different darkness respond to bacterial degradation has not previously been assessed. All melanin-containing feathers have eumelanin and pheomelanin granules, and differences in darkness might arise through the amount and relative proportion of the two pigment types (McGraw 2006) or through the arrangement of the melanin granules in the barbules (Shawkey *et al.* 2006b).

The Shiny Cowbird *Molothrus bonariensis* is a sexually dimorphic species, in which males show a black iridescent plumage, compared with the dull brown plumage of females. This plumage pattern is found across almost the entirety of its distribution, except in northern Argentina, eastern Paraguay and southern Brazil (Friedmann 1929). In these areas, females show polymorphic plumage, with colours ranging from dull brown to dark brown and black.

The aim of this study was to test whether increased darkness in Shiny Cowbird females in the subtropics confers increased protection against feather-degrading bacteria. We therefore compared the degradation of female feathers belonging to different colour morphs from subtropical and temperate zones when exposed to bacterial strains isolated from both areas. If dark feathers were more effective in resisting bacterial degradation this could be an explanation for the observed plumage pattern. To our knowledge this is the first time that bacterial degradation has been assessed on melanin-containing feathers in a species showing polymorphic plumage colours.

METHODS

Feather samples

We mist-netted adult Shiny Cowbirds during the months of August and September 2006 in two different locations within Argentina, during the winter when bacterial load is at its maximum (Burtt & Ichida 1999). The first location, Punta Indio, Buenos Aires Province (35°16′S, 57°15′W), lies within the temperate zone where only dull brown females are found, whereas Reserva El Bagual, Formosa Province (26°10′S, 58°56′W), is within the subtropical region where dark-coloured females are present. The first location has an annual mean temperature of 14.8 °C, a mean humidity of 77% and an annual precipitation of 900 mm; at the second locality values reach 22 °C, 76% and 1456 mm, respectively.

In Buenos Aires we captured 24 males and 10 females while in Formosa we captured 19 males and 22 females (10 dull brown, six dark brown and six black). Before handling each bird, we disinfected our hands and allowed them to air-dry. We plucked approximately 10 ventral feathers from each bird and placed them in a sterile plastic envelope for further analysis. Envelopes were kept at 4 °C for no longer than 1 week.

Isolation of bacteria

To isolate bacterial strains and study bacterial load we analysed the feathers of 30 individuals from each locality, belonging to 21 males and nine

females captured in Buenos Aires, and eight males and all 22 females captured in Formosa. Bacteria present on the external side of four feathers from each individual were recovered by imprinting feathers for 30 s on a tryptic soy agar (TSA) plate divided in quadrants. Feathers were similar in size within individuals and between individuals, both of different sex and from different localities. Plates were incubated for 24 h at 30 °C. After this period of time we counted the colonies present in each quadrant and calculated a mean value of bacteria per feather for each individual as well as the coefficient of variation as a measure of repeatability. This allowed us to compare bacterial load between sexes and localities using a two-way ANOVA. Values were log-transformed for this analysis.

We randomly selected three plates per locality, choosing at least one male and one female, and picked 15 colonies from each plate at random with sterile applicators. Each applicator was placed in an individually labelled, sterile tube of nutrient broth supplemented with 7.5% NaCl. Tubes were incubated at 48 °C for 7 days to select for feather-degrading bacteria, which are favoured by high temperatures and saline broth (Burtt & Ichida 1999). If the broth became cloudy, the bacteria were cultured by streaking a loopful of the medium on TSA plates and incubated at 30 °C for 48 h. The morphology of the resulting colonies was checked to determine the purity of the culture for further analyses.

Bacterial isolates were tested for feather-degrading activity, for which we randomly picked 10 of the isolates recovered from each locality. Flasks containing 10 mL of feather medium (Williams *et al.* 1990) and 0.05 g of white secondary remiges from Coscoroba Swans *Coscoroba coscoroba* were sterilized by autoclaving. Although this sterilization method may not be optimal (Gunderson *et al.* 2008), we are confident that it did not affect the outcome of the experiments as all feathers were treated the same way. Each bacterial isolate was grown overnight on a TSA plate and bacterial colonies were resuspended in 3 mL of feather medium to inoculate the flasks to an initial optical density at 600 nm (OD_{600}) of 0.05. One flask was inoculated with *B. licheniformis* strain OWU 138B as a positive control and another flask was not inoculated with any strain as a negative control. Cultures were incubated for 1 week at 30 °C with constant shaking. After this period of time, a 1-mL aliquot of each flask was removed and centrifuged at 16 000 ***g*** for 5 min. We placed the supernatant into quartz cuvettes and measured absorbance at 230 nm with a Shimadzu UV-1603 spectrometer. Absorbance values are proportional to oligopeptide fragments of β-keratin released by feather degradation (Goldstein *et al.* 2004). Degradation of feathers was also assessed by eye.

We also tested for differences in feather-degrading intensity between strains at both localities by repeating the above experiment (0.05 g of white feathers in 10 mL of feather medium inoculated with an initial OD_{600} of 0.05) for six feather-degrading strains from each locality and by taking 0.5-mL aliquots after 24, 48, 72, 144 and 216 h. Aliquots were diluted 10 times, as absorbance for the undiluted samples was maximal after the first 24 h. Degradation was compared using repeated-measures ANOVA.

Bacterial degradation experiments

Further experiments of feather degradation were undertaken with *B. licheniformis* and two native feather-degrading strains, one from each locality, selected at random. Each flask used in the experiments contained 5 mL of feather medium and the tips of four ventral feathers plucked from one individual. We only included the coloured part of the feather, discarding the proximal part. After autoclaving, flasks were inoculated with 0.05 OD_{600} of each particular strain and incubated at 30 °C with constant shaking for 1 week. For each experiment, we studied female dull brown and black feathers using six replicates (i.e. from six different individuals). For dull brown feathers we used three belonging to females captured in Formosa and three belonging to females captured in Buenos Aires. We also included three intermediately coloured dark brown feathers and three flasks containing Coscoroba Swan white feathers in an amount similar to the ventral feathers of Shiny Cowbirds to control for bacterial activity. An additional flask of each feather morph was not inoculated with bacteria and used as negative control. After 1 week, absorbance was measured at 230 nm as described above. To test for differences in degradation, we subtracted control values of each colour morph from absorbance values of those feathers. This allowed us to control for differential responses to incubation by feather type (Gunderson *et al.* 2008). Differences in degradation were tested using two-way ANOVA, with feather colour and bacterial strain as

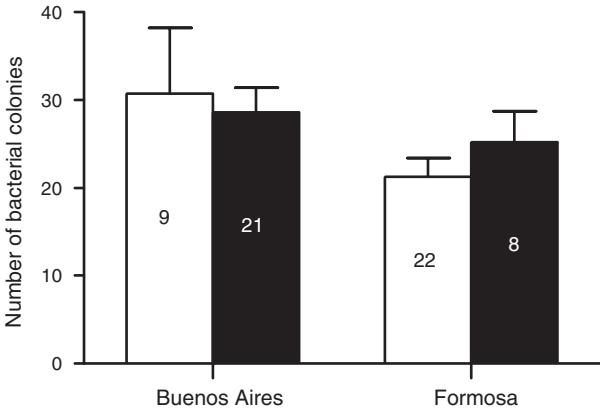

Figure 1. Number of bacterial colonies in feathers plucked from male (black) and female (white) Shiny Cowbirds from Buenos Aires (temperate) and Formosa (subtropical). Numbers inside bars indicate sample size.

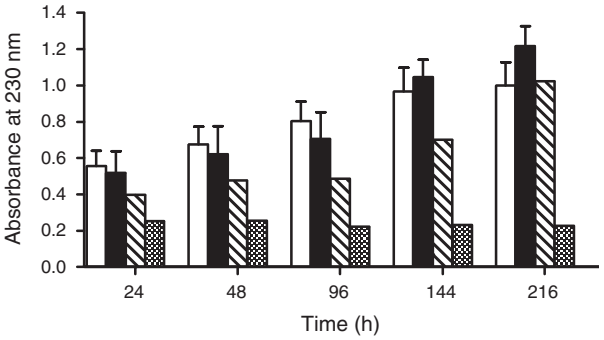

Figure 2. Values correspond to absorbance at 230 nm by media containing dissolved oligopeptides of white feathers degraded by bacterial strains isolated from feathers of Shiny Cowbirds trapped in Buenos Aires (white bars, $n = 6$ feather-degrading strains) and Formosa (black bars, $n = 6$ feather-degrading strains), and by *B. licheniformis* strain 138B (striped bars). The control medium (dotted bars) did not contain bacteria.

factors. For post-hoc pairwise comparisons we used the unequal N HSD-test. Data were transformed as follows: 1/(absorbance + 1) (Zar 1996).

RESULTS

Bacterial load and bacterial activity

We found no significant difference in bacterial load between feathers from different localities ($F_{1,56} = 2.96$, $P = 0.09$; Fig. 1) and feathers plucked from individuals of different sex ($F_{1,56} = 0.50$, $P = 0.48$; Fig. 1). Interaction between predictor variables (locality and sex) was not significant ($F_{1,56} = 0.90$, $P = 0.34$). The mean coefficient of variation among measurements of feathers of the same individual was 36% (range: 10–68%).

Of the 15 colonies randomly picked from three plates from each locality, approximately the same number of bacteria grew for all individuals under selective conditions of salinity and temperature (mean ± se: Buenos Aires = 7 ± 1.52 strains; Formosa = 7.33 ± 1.45 strains), resulting in a total of 21 strains isolated from Buenos Aires and 22 strains isolated from Formosa. Of the 10 strains from each locality tested for feather-degrading activity, eight from Buenos Aires and six from Formosa showed maximal absorbance at 230 nm after 1 week. These bacteria transformed white feathers into minute pieces of rachis or barbs just visible in the solution, whereas for bacteria that did not degrade, absorbance was similar to the negative control and feathers looked undamaged. The intensity of feather-degrading activity did not differ between strains isolated from the different localities (repeated-measures ANOVA: $F_{5,12} = 0.53$, $P = 0.74$; Fig. 2).

Bacterial degradation experiments

Feather degradation did not differ between female dull brown feathers coming from different localities ($F_{1,12} = 0.10$, $P = 0.74$). We thus pooled them when analysing the differences in degradation between feather morphs.

Degradation did not differ between strains ($F_{2,36} = 1.71$, $P = 0.19$) but differed significantly between feather morphs ($F_{3,36} = 23.34$, $P < 0.0001$; Fig. 3). Pairwise comparisons showed that the significant differences only involved white feathers, for which the absorbance differed significantly from bacterial solutions of female Shiny Cowbird feathers of all colour morphs ($P < 0.001$). However, Shiny Cowbird feathers belonging to different morphs did not differ significantly from each other ($P > 0.07$). When measurements were taken after 1 week, white feathers were completely degraded, whereas melanized feathers looked undamaged.

DISCUSSION

We did not find differences in bacterial load and bacterial feather-degrading activity between a subtropical location where Shiny Cowbird females with polymorphic plumage are present and a

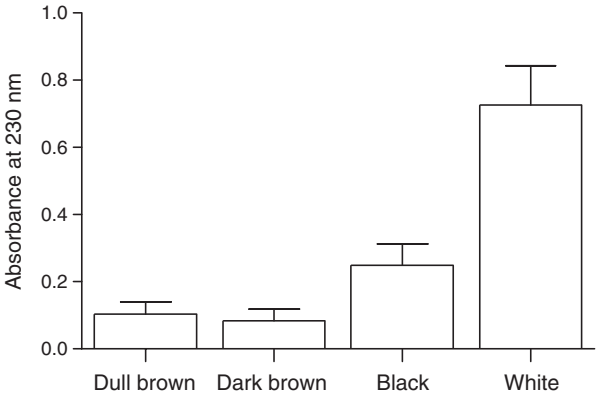

Figure 3. Values correspond to absorbance at 230 nm by media containing dissolved oligopeptides of feathers of different colour minus its respective control after 1 week of exposure to feather-degrading activity by three different bacterial strains. Numbers of replicates for dull brown, dark brown, black and white feathers were: 14, 8, 17 and 9, respectively.

temperate location where only dull brown females are found. For both localities, bacterial load on feathers and feather-degrading activity of isolated bacterial strains were similar. We found no evidence to suggest that increased darkness of feathers offers greater protection against bacterial degradation. The feather polymorphism of Shiny Cowbird females thus does not seem to have arisen as a response to feather degradation by bacteria.

Previous evidence of feather degradation by bacteria demonstrated that melanized feathers were more resistant to bacterial activity compared with white feathers (Goldstein *et al.* 2004, Gunderson *et al.* 2008, but see Grande *et al.* 2004). In this study, we also found that after 1 week of bacterial degradation white feathers were reduced to dust, whereas feathers with melanin content remained practically unaffected, retaining the shape they showed at the beginning of the experiment. Our results suggest that it is the presence of melanin that accounts for the resistance to bacterial activity, but not the darkness of the feathers. Feathers of different colour morphs might be equally protected against bacteria-degrading activity due to similar melanin content that varies in the relative amounts of pheo- and eumelanins (McGraw 2006) or in the arrangement of melanin granules in the feathers (Shawkey *et al.* 2006b). Alternatively, if different feather morphs contain variable amounts of melanins, it might be the sole presence of melanins, but not the concentration, that confers resistance to bacterial degradation. In an experiment in which melanized feathers of two bird species were exposed to bacterial degradation *in vivo*, there was no damage compared with feathers without bacteria (Cristol *et al.* 2005). However, in this experiment, preening or sunning behaviour might have reduced the effects of bacterial feather-degrading activity on the feathers.

Darker plumage in populations living in humid areas has been shown for many avian species (Zink & Remsen 1986) and is known as Gloger's rule (Gloger 1833). Although we could not find evidence supporting the notion that the increased darkness of Shiny Cowbird feathers protects them to a greater extent against bacterial degradation, a correlation of bacterial feather-degrading activity and plumage darkness has been shown for two sparrow species (Burtt & Ichida 2004, Peele *et al.* 2009). Feather degradation would impose a selective pressure on the plumage that varies according to ecological factors influencing bacterial activity; for example, more humid and salty areas favour bacterial growth. As a consequence, populations inhabiting areas with stronger selective pressures would acquire darker plumage. Different susceptibilities to bacterial feather-degrading activity among species might arise from pigment type or arrangement. Since eumelanins colour feathers brown and black and pheomelanins colour them primarily rufous (McGraw 2006), Shiny Cowbirds' feathers are most probably coloured by the first (Shawkey *et al.* 2006b). It is possible that the brown plumage of sparrows has a different basis, making feathers sensitive to bacterial activity. Alternatively, sexual selection might be playing a role. Recent studies (Shawkey *et al.* 2007, 2009, Gunderson *et al.* 2009) have shown that feather-degrading bacteria may have some role in sexual selection by influencing the expression of a secondary sexual trait. Plumage colour is affected by keratinolytic activity of bacilli and might signal abundance of feather-degrading bacteria to potential mates. Hence, plumage coloration can be an indicator of bacterial load and thus of individual quality. The non-overlapping polymorphic populations of sparrows might have arisen from sexual selection favouring darker plumage that signals better condition in areas with higher bacterial activity. However, to test for sexual selection, preferences of individuals and the effects of bacterial degradation on plumage colour have to be assessed.

In summary, we did not find evidence supporting the hypothesis that increased darkness in female Shiny Cowbird's feathers offers greater protection against bacterial degradation. Instead, it seems that the sole presence of melanins acts as a barrier against feather-degrading bacteria.

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