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VARIATION IN ASSEMBLAGES OF FEATHER BACTERIA IN RELATION TO PLUMAGE COLOR IN FEMALE GREAT TITS

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Abstract. Microorganisms are known to play an important role in shaping the life histories of animals. Recent studies have proposed that the coloration of birds' plumage could reflect individual quality through associations with feather-degrading bacteria. However, few studies have explored such relationships. We studied breeding female Great Tits (*Parus major*) during nest building and chick rearing to explore associations between bacteria inhabiting their yellow chest feathers and feather coloration. Specifically, we used flow cytometry and ribosomal intergenic spacer analysis (RISA), respectively, to study the densities of all free-living and attached bacteria and the phylotypic richness of feather-degrading bacterial assemblages. We used chroma (color saturation) as a measure of feather coloration. During chick rearing but not during nest building, the female's chroma was negatively related to the phylotypic richness of feather-degrading bacteria. Also, a seasonal change in the density of attached bacteria associating with individual birds was negatively associated with change in chroma over the same period. These findings suggest that conspicuous coloration of female Great Tits may reflect the numbers and character of bacteria inhabiting feathers.

Key words: *carotenoid-based coloration, chroma, feather-degrading bacteria, female coloration,* Parus major.

Variación en el Ensamble de Bacterias de las Plumas con Relación al Color del Plumaje de la Hembra de *Parus major*

Resumen. Se sabe que los microorganismos juegan un rol importante en modelar las historias de vida de los animales. Estudios recientes han propuesto que la coloración del plumaje de las aves podría reflejar la calidad individual indicando la asociación con bacterias que degradan las plumas. Sin embargo, pocos estudios han explorado estas relaciones. Estudiamos hembras reproductivas de *Parus major* durante la construcción del nido y la cría de pichones para explorar asociaciones entre bacterias que habitan sus plumas amarillas del pecho y la coloración de las plumas. Específicamente, usamos citometría de flujo y análisis ribosomal de espaciadores intergénicos (RISA por sus siglas en Inglés), respectivamente, para estudiar las densidades de todas las bacterias libres y ligadas y la riqueza filotípica de los ensambles de bacterias que degradan las plumas. Empleamos croma (saturación del color) como una medida de coloración de la pluma. Durante la cría de los pichones pero no durante la construcción del nido, la croma de la hembra estuvo negativamente relacionada a la riqueza filotípica de las bacterias que degradan las plumas. Además, el cambio estacional en la densidad de las bacterias ligadas asociadas con aves individuales estuvo negativamente asociado con los cambios en la croma a lo largo del mismo período. Estos resultados sugieren que la coloración conspicua de las hembras de *P. major* puede reflejar la cantidad y el carácter de las bacterias que habitan las plumas.

INTRODUCTION

Bird plumage is inhabited by an assemblage of bacterial taxa (Burtt and Ichida 1999, Bisson et al. 2007, Gunderson 2008), some of which are capable of degrading β-keratin, a protein that constitutes more than 90% of feather mass (Burtt and Ichida 1999, Sangali and Brandelli 2000, Lucas et al. 2003b). Such feather-degrading bacteria may play an important role in shaping the life histories of their avian hosts (Burtt and Ichida 1999, 2004, Shawkey et al. 2007, Peele et al. 2009).

Several authors have suggested that feather-degrading bacteria could influence feather-based communication by modifying feather coloration. In the Eastern Bluebird (*Sialia sialis*), for example, Shawkey et al. (2007) and Gunderson et al. (2009) found that structural color is affected directly by feather-degrading bacteria. Shawkey et al. (2009) have also explored the association between carotenoid-based coloration and feather-degrading bacteria in male House Finches (*Carpodacus mexicanus*).

Models of sexual selection generally propose that ornamental traits of animals signal certain aspects of the male's

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quality (reviewed in Andersson 1994). One explanation for the evolution of such traits is that their expression is a signal of resistance to various parasites (Hamilton and Zuk 1982, reviewed in Moore and Wilson 2002). However, in many bird species, both sexes are conspicuously colored, though the coloration is usually expressed to a lesser extent in females (Kraaijeveld et al. 2007). Reflecting this, interest in the function and evolution of female ornaments has recently been increasing (reviewed in Amundsen 2000, Kraaijeveld et al. 2007, Clutton-Brock 2009).

Plumage coloration can also change between molts (e.g., McGraw and Hill 2004, Figuerola and Senar 2005). Several mechanisms, including abrasion (Burtt 1986, Willoughby et al. 2002), sun bleaching (Surmacki 2008), presence of preen oil (Pérez-Rodríguez et al. 2011), and dirt accumulation (Surmacki and Nowakowski 2007) are known to bring about such changes. However, only one study (Gunderson et al. 2009) has explored the association between the activity of feather-degrading bacteria and seasonal color changes in individual birds. It has also been suggested that an association between plumage color and the abundance of bacteria in the plumage may emerge if both traits are related to some aspect of individual quality, such as effort in preening (Shawkey et al. 2009)

In this study we examined two aspects of the plumage of the female Great Tit (*Parus major*): on one hand, plumage coloration, on the other, the density of free-living and attached bacteria and the phylotypic richness of feather-degrading bacteria. In the Great Tit, both males and females have conspicuous yellow chest plumage, and this carotenoid-based color reflects aspects of individual quality in both sexes (Hõrak et al. 2001, Mänd et al. 2005a, Senar et al. 2008, Broggi and Senar 2009). In a natural environment, however, this yellow can also be cryptic to predators (Delhey et al. 2010). Thus the degree of color expression in Great Tits and similar species may be regulated by a combination of sexual and natural selection.

We investigated whether the plumage chroma (color saturation) of adult female Great Tits is correlated with bacterial colonization of the plumage and whether there is a correlated seasonal change in these two traits within an individual. On the basis of earlier findings that higher chroma (Senar et al. 2008, Broggi and Senar 2009) and lower density of attached bacteria (Saag et al. 2011) signal better individual quality of female Great Tits, we predicted a negative association between chroma and bacterial density.

METHODS

GENERAL FIELD METHODS

Our study area, near Kilingi-Nõmme (58° 7′ N, 25° 5′ E) in southwestern Estonia, covers approximately 50 km^2 and contains a mosaic of coniferous and deciduous forest (see a map of the study area in Mänd et al. 2005b).

Great Tits bred in wooden nest boxes with a cavity of 11 × 11 × 30 cm and an entrance diameter of 3.5–4.0 cm. Distances between neighboring nest boxes were 50–60 m. Nest boxes were cleaned to remove old nest material before the beginning of the breeding season. In 2007, we captured females during nest building, before the start of egg laying. All females abandoned their nests after this initial capture, but most made another nest in a neighboring box. We captured the females again when their nestlings were 10–14 days old. The mean time between the two captures was 40 days (range 33–53 days). The number of days elapsed between the two captures was unrelated to either seasonal changes in chroma or bacterial traits (Spearman correlation, all $P > 0.4$). Both times we trapped the females, we weighed them with a Pesola spring balance to a precision of 0.1 g and, using digital calipers, measured their tarsi to the nearest 0.1 mm. In total, we sampled 52 females before laying and 40 during nestling rearing, among them 12 sampled during both periods.

DENSITY OF FREE-LIVING AND ATTACHED BACTERIA ON FEATHERS

We handled each female with a fresh pair of examination gloves. Within 30 sec after capture, we removed approximately five ventral feathers from the center of the yellow chest to determine the densities of bacteria and another five to determine the phylotypic richness of feather-degrading bacteria. Both samples were placed into dry, clean microtubes with forceps sterilized in 96% ethanol, immediately stored at 4°C, and transported in a cool box to the laboratory, where they were stored at –80° C prior to analysis. Two distinguishable ecological types of bacteria inhabit bird plumage: free-living and attached bacteria (Lucas et al. 2005). Studies of bacterial communities in soil, water, and sediment have demonstrated that free-living bacteria are usually more labile, while attachment provides a more stable environment and protection against grazing, chemical antibiotics, and physical forces (see Lucas et al. 2003a, Selje and Simon 2003 for references). Following Lucas et al. (2003a), we separated the two types of bacteria in the laboratory and, to determine their density, counted free-living and attached bacteria directly with a flow-cytometry machine (BD LSR II). For tagging we used the DNA-binding dye SYBR Green (for further details see Saag et al. 2011). Bacterial densities are expressed per feather.

PHYLOTYPIC RICHNESS OF FEATHER-DEGRADING BACTERIA

To determine the phylotypic richness of feather-degrading bacteria, we covered sampled feathers with buffer solution (1.5 mL of phosphate-buffered saline) then incubated them in this buffer for 30 days at 26°C in the dark without using a shaker (following Lucas et al. 2005). As feathers were the only source of carbon in the enrichment medium, presumably only bacteria capable of degrading keratin were promoted. There remains the possibility that certain non-keratin-degrading

species occurred at extremely high densities before enrichment of samples or still managed to procreate during incubation and that the DNA of these species could have been picked up in the analysis even after the enrichment procedures. However, taking into account the low mean number of phylotypes per bird (see Saag et al. 2011), we expect the probability of such species present in the later analyses to be low in comparison with that of feather-degrading species. To analyze the structure of feather-degrading bacterial assemblages obtained in the enrichment cultures we used the ribosomal intergenic spacer analysis (RISA) method (Ranjard et al. 2000a,b). Each RISA band is assumed to correspond to one bacterial species; following Muyzer et al. (1993) and Stach et al. 2003), we refer to such species as phylotypes. Thus the number of bands corresponds to the richness of the bacterial assemblage (Ranjard et al. 2000b). We separated amplified products by electrophoresis on a 3% agarose gel for 1 hr at 140 V. Band profiles were photographed and aligned by eye (in Adobe Photoshop). In each sample we recorded the bands present and estimated bacterial richness as the total number of phylotypes (for details see Saag et al. 2011).

COLOR MEASUREMENTS

For color measurements we plucked three additional feathers from the center of the yellow breast of each individual. To characterize breast color we measured the values of chroma with a spectrophotometer (Ocean Optics USB2000 with Ocean Optics DH2000 lamp). Chroma corresponds to color purity with higher values representing more pure color (Endler 1990). The chroma of the yellow breast feathers of female Great Tits is related to nest size (Broggi and Senar 2009) and body condition (Senar et al. 2008). We measured chroma in the visible range of 400–700 nm (Senar et al. 2008, Broggi and Senar 2009), placing the three feathers on top of each other. We measured each sample three times and used the mean in analyses. The within-individual repeatability of the three chroma measurements was high ($r = 0.86$, $F_{44, 90} = 20.1$, $P < 0.001$; calculated according to Lessells and Boag 1987).

STATISTICAL ANALYSES

For statistical analyses we used Statistica 10.0 (StatSoft). To analyze the effects of bacterial traits on chroma we used a general linear model (GLM). To calculate the seasonal change in chroma and bacterial traits, we subtracted the values measured before egg laying from those measured during chick rearing. Because of the relatively small sample sizes, we used nonparametric (Spearman) correlation to analyze the associations between seasonal changes in chroma and bacterial traits. For the GLM, the density of attached bacteria was square root transformed and the density of free-living bacteria was ln transformed before analyses to achieve normality. We used a standardized regression coefficient (β) to describe the size of the effect of continuous predictor variables in the prediction of the dependent variable. When analyzing the effect of number of nestlings on chroma, we excluded deserted or depredated nests from the analyses.

RESULTS

Before laying there were no associations between chroma and bacterial traits (Table 1). During the nestling-feeding period, feather chroma was negatively correlated with the phylotypic richness of feather-degrading bacteria ($\beta = -0.38$; Table 1, Fig. 1). This result did not change if nonsignificant factors were removed from the model. Chroma was not related to the densities of either attached or free-living bacteria (Table 1). These results did not change if the female's body mass, body condition (body mass corrected for tarsus length), number of nestlings, forest type, or capture date were included in the models; however, these factors were always nonsignificant and were therefore removed from the final models.

Change in feather chroma between the pre-laying and chick-rearing periods was negatively correlated with change in density of attached bacteria during the same period (Spearman correlation, $r_s = -0.82$, $P = 0.001$, $n = 12$; Fig. 2). Accordingly, in individuals whose densities of attached bacteria increased over time, feather chroma decreased, and vice versa. Seasonal changes in feather chroma were not associated with changes in the density of free-living bacteria or phylotypic richness of feather-degrading bacteria (all $P > 0.5$). Seasonal changes in chroma or bacterial traits were not correlated with seasonal changes in body mass (all $P > 0.5$).

DISCUSSION

We found that feather chroma was negatively related to the phylotypic richness of feather-degrading bacteria in females' plumages during the chick-rearing but not the pre-laying period. At the same time, we found no associations between chroma and bacterial densities during either of the sampling

TABLE 1. The effects of plumage bacteria (densities of free-living and attached bacteria and phylotypic richness of feather-degrading bacteria) on the chroma of the yellow ventral feathers of female Great Tits. The results of a multiple regression (GLM) are shown.

Breeding phase and predictor variable	df	*F*	*P*
Pre-laying period			
Density attached	1	0.0	0.97
Density free-living	1	0.38	0.54
Phylotypic richness	1	1.72	0.19
Error	48		
Brood-rearing period			
Density attached	1	1.05	0.31
Density free-living	1	0.93	0.34
Phylotypic richness	1	6.19	0.018
Error	36		

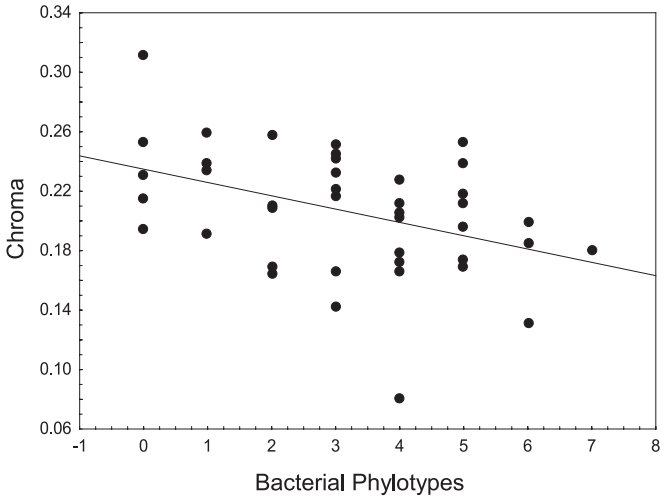

FIGURE 1. Association between chroma and feather-degrading bacterial phylotypic richness in the yellow ventral feathers of female Great Tits during the chick-rearing period.

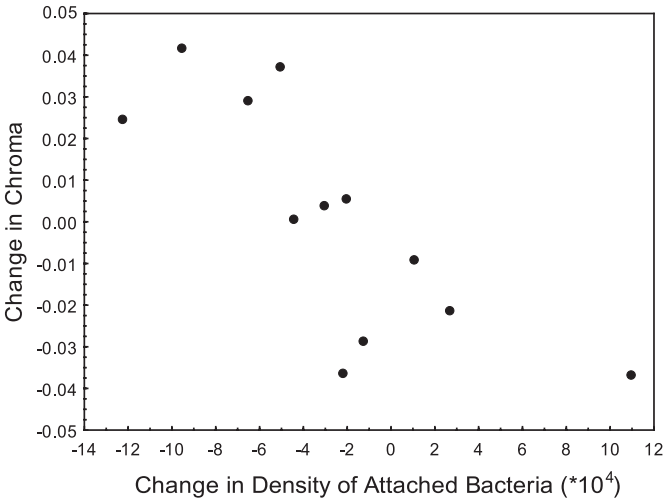

FIGURE 2. Association between seasonal changes in the density of attached bacteria and the chroma of yellow ventral feathers of female Great Tits. For both traits positive values indicate a seasonal increase, negative values a seasonal decrease.

periods. However, seasonal change in densities of attached bacteria was correlated with change in feather chroma, so that an increase in this density was accompanied by a decrease in chroma and, conversely, a decrease in bacterial density was accompanied an increase in chroma.

This is the first study to have explored associations between carotenoid-based coloration and plumage bacteria in female birds. The only previous study examining the associations between carotenoid-based plumage coloration and plumage bacteria found that male House Finches with redder plumage had lower loads of feather-degrading bacteria (Shawkey et al. 2009). From our results, we suggest that the chroma of the yellow chest of female Great Tits may indeed signal some aspect of individual quality (see also Senar et al. 2008, Broggi and Senar 2009). Previous studies have found that feather chroma is more sensitive to developmental perturbations and body condition than is feather hue (Shawkey et al. 2003, Senar et al. 2008). Senar et al. (2008) also found feather chroma of the Great Tit to be unrelated to the carotenoid content of the feather, indicating that other mechanisms may also be responsible for variations in this trait (but see Isaksson et al. 2008).

Because of the correlative nature of our results, it remains to be clarified whether plumage bacteria affected plumage color directly or if both were in fact correlated with a third factor. The mechanisms by which feather-degrading bacteria influence carotenoid-based color are currently the subject of debate. In the Eastern Bluebird, feather-degrading bacteria can affect structural plumage coloration directly (Shawkey et al. 2007, Gunderson et al. 2009). It is thus possible that feather-degrading bacteria affect the microstructure that is also involved in producing carotenoid-based coloration (Shawkey and Hill 2005). It is also possible that feather-degrading bacteria could damage carotenoid structure (as suggested by McGraw and Hill 2004). However, Burtt and Ichida (1999) have also suggested that molt may decrease bacterial load on feathers (but see Giraudeau et al. 2010). At the same time, the chroma of new feathers may differ from that of old feathers, reflecting nutritional conditions during the molt (e.g., Hill 2000). As Great Tits start molting at the end of the breeding season, this process can sometimes overlap with the rearing of late broods (e.g., Orell and Ojanen 1980). We captured females before they laid, and this resulted in desertion of nests and presumably also in delayed egg laying in the new nests built by females. Therefore we cannot exclude the possibility that the negative association between bacterial traits and plumage color was caused by some breeding individuals starting to molt earlier than others and thus affecting both traits simultaneously. Second, the coloration of many species' plumage changes between molts as a result of bleaching, dirt accumulation, presence of preen oil, and abrasion (see Introduction). For example, in the Bohemian Waxwing (*Bombycilla garrulus*) both addition of preen oil and removal of soil increased the chroma of the yellow tips of the tail feathers (Pérez-Rodríguez et al. 2011). Such changes can also vary on an individual basis, so that, during the same time period, values for hue and chroma increase in some birds but decrease or remain constant in others (McGraw and Hill 2004). It is thus possible that some individuals are better able than others to care for their plumage, thus affecting both plumage coloration and the communities of feather-inhabiting bacteria.

In contrast to the pattern described above, we did not find any association between plumage chroma and bacterial traits during the pre-laying period. Previously we have found marked seasonal changes in bacterial densities in the plumage of the Great Tit (Saag et al. 2011). The present study suggests that the association between plumage color and feather bacteria also varies seasonally.

In conclusion, we have shown that yellow chest coloration of brood-rearing female Great Tits is negatively associated with the phylotypic richness of feather-degrading bacteria and that there are parallel seasonal changes in color and density of attached bacteria. These findings suggest that conspicuous coloration of female Great Tits may signal the numbers and character of bacteria inhabiting feathers. However, experimental studies are needed to test these associations' causality and potential linkage with individuals' quality.

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