Letters

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Feather microstructure predicts size and colour intensity of a melanin-based plumage signal

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Feather microstructure affects the light absorbed by plumage pigments. However, the effect of particular elements of feather microstructure on the expression of pigmentary colours or on the size of colour patches has never been investigated. Here I use a model of avian visual perception and scanning electron microscope imaging of feathers to show that part of variation in the size and colour properties of a melanin-based plumage signal of quality, the black breast stripe of great tits *Parus major*, is explained by three elements of feather microstructure (barbule density, barb cortex size and barb pith size). The strongest associations were between large stripes and low barbule density, between dark stripes and high barbule density, and between stripes with high relative long reflectance and high barbule density and thin barb cortex. By contrast, carotenoid-based colour was not related to microstructural elements. Thus, it is possible that not all variation in melanin-based colour is determined by melanin content, but also by feather microstructure. These findings should be considered by studies on the evolution of signals of quality.

Plumage colours are produced by the differential absorption of light by pigments (pigmentary colours) or by the interaction of light waves with specialized microstructures of feathers (structural colours) (Shawkey et al. 2005). However, pigmentary colours also depend on feather microstructure (Shawkey and Hill 2005). While the mechanisms of structural colour production are relatively well known (Shawkey et al. 2003, 2005, Doucet et al. 2004, 2006), the mechanisms by which feather microstructure affects pigmentary colours are limited to the effect of light reflected by the keratin of feathers on carotenoid-based colour (Shawkey and Hill 2005). The particular elements of feather microstructure that affect the expression of pigmentary colours, as well as their potential interaction with pigments different from carotenoids, remain poorly known.

Here I explore the interaction between some elements of feather microstructure and the expression of pigmentary colours in great tits *Parus major*. I focus on the density of barbules, the size of the barb keratin cortex and the size of the barb pith (i.e. the space comprising the medullary spongy layer of keratin and air), three elements that have already been shown to affect the expression of structural colour (Shawkey et al. 2005). I investigate the potential effects of these elements on the colour of a melanin-based trait (the black breast stripe) and two carotenoid-based traits (the yellow breast and green back patches) (Fig. 1a). These colour patches can be perceived as a multiple condition-dependent signal (Galván 2010). I also investigate the potential effect of feather microstructure on the size of the black breast stripe, a well known melanin-based signal present in both sexes that is positively related to social dominance and reproductive success (Järvi and Bakken 1984, Norris 1990, Carrascal et al. 1998, Quesada and Senar 2007). Therefore, any effect of feather microstructure on these colour traits would be of key importance for understanding the evolution of honest signals generated by pigments, as the production of feather microstructures may impose certain costs only affordable by high quality individuals (Galván 2011). To determine whether these potential effects are significant in a context of intraspecific signalling, I use a model of avian visual perception.

Methods

Sampling

The study was carried out in January–March 2006 in a deciduous forest of Pyrenean oak *Quercus pyrenaica* in Miraflores de la Sierra, central Spain (40°49′N, 03°46′W). A total of 44 great tits (36 males and eight females, representing 33 adults, 10 yearlings and one individual of unknown age) were captured at artificial feeders with funnel-traps. A digital calliper was used to measure the tarsus length of birds and the size of the black ventral stripe by measuring the minimum width to the nearest 0.01 mm.

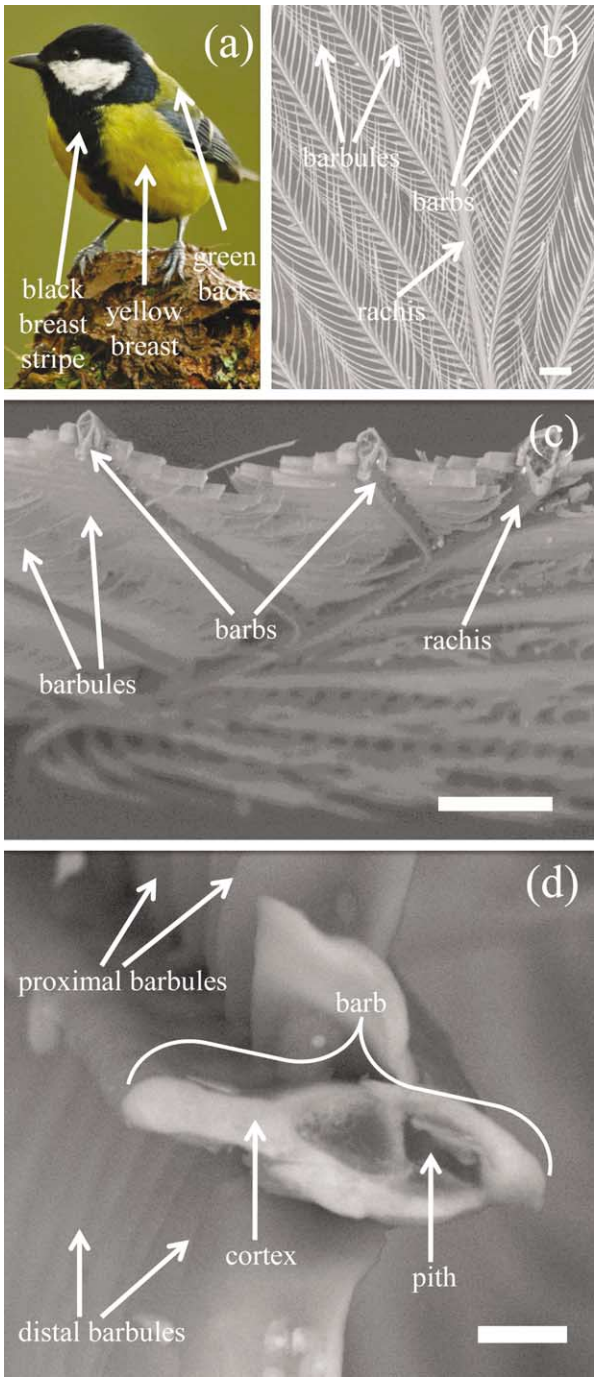

Figure 1. (a) An adult male great tit (courtesy of Rafael Palomo Santana). (b) SEM micrograph of a longitudinal section of a feather (scale bar = 100 μm). (c) SEM micrograph taken on a feather sample in an upright position (scale bar = 100 μm). (d) SEM micrograph of a cross-section of a barb (scale bar = 10 μm).

I chose this measure for the stripe size because it is well correlated with the total area of the stripe and can be taken with a low measuring error (Senar 2004). The stripe size was measured without modifying the natural position of the feathers, and maintaining the birds in a standard position. The thinnest part of the stripe was always located in approximately the same region of the birds. The variance of the stripe size was 1.87 in males and 0.30 in females.

The birds were weighed on a portable electronic balance to the nearest 0.1 g. Body condition was calculated as body mass divided by the cube of tarsus length (Galván 2010). The three most external feathers at the points where plumage colour was measured were plucked. The total number of birds with available information on morphometrics, plumage colour and feather microstructure was 33. Following Shawkey et al. (2005), data from males and females were pooled to consider more extensive variation than if only data from one sex were used instead, and thus conferring to the analyses a greater capacity to elucidate the relationships between colour and feather microstructure. However, the results obtained excluding females from the analyses are also shown.

Reflectance spectrometry

Plumage reflectance measurements from the three plumage patches were collected in the field using an Ocean Optics USB2000 spectrophotometer (range 250–800 nm) with UV (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400-μm fibre-optic probe (Ocean Optics, Dunedin, FL, USA). The fibre-optic probe both provided illumination and obtained light reflected from the sample, and had a reading area of ca 1 mm^2. The measurements were taken at a 90° angle to the sample. The spectrometer measured reflectance in 0.36-nm increments. All measurements were relative to a white Spectralon tablet (WS-1-SS; Ocean Optics), and the system was frequently calibrated. The spectral curves were generated using OOIBASE software. Three readings were obtained at different points of each plumage patch, moving the probe by at least 5 mm before taking each new reading, but always following the same order (from upper to lower patch) and taking each reading from approximately the same location in all birds. The results of reflectance measurements are shown elsewhere (Galván 2010).

Visual modelling

Colour vision in diurnal birds is dependent upon four types of retinal cone cells which are sensitive to either very short (VS, ultraviolet (UV)), short (SWS, blue), medium (MWS, green) or long (LWS, red) wavelengths (Maier and Bowmaker 1993). Spectral data were reduced into four quantal cone catches (Vorobyev et al. 1998) that quantify the amount of light captured by each of the single cones. By using the SPEC package (Hadfield and Owens 2006) implemented in R statistical environment, the four quantum cone catches (Q_i) were calculated by multiplying cone sensitivities by the reflectance spectrum, the irradiance spectrum and the transmission spectrum of the ocular media, as expressed by the formula:

$$Q_i = \frac{\int_{\lambda} R_i(\lambda)S(\lambda)I(\lambda)O(\lambda)d(\lambda)}{\int_{\lambda} R_i(\lambda)}$$

where λ indicates wavelength (nm), $R_i(\lambda)$ is the sensitivity (nm) of cone type i, $S(\lambda)$ is the percent of light reflected

from a patch compared to a white standard, $I(\lambda)$ is the irradiance spectrum ($\mu mol \cdot m^{-2} s^{-1} \cdot nm^{-1}$) and $O(\lambda)$ is the transmittance spectrum of the ocular media.

As cone catches change with varying illumination, $I(\lambda)$, and this is discounted by animals through colour constancy, the von Kreis algorithm was used to normalize Q_i by the quantum cone catch for the irradiance spectrum. The irradiance spectral data used were obtained from a standard forest shade illumination provided by the SPEC package (Hadfield and Owens 2006), as this irradiance spectrum is the most appropriate for the present case, and an achromatic adapting background (Hadfield and Owens 2006). I used the ocular media transmittance of a species closely related to the great tit (i.e. the blue tit *Cyanistes caeruleus*; Hart et al. 2000). Each cone catch was then standardized by dividing them by the sum of the four cone catches, and three of the standardized cone catches were divided by a fourth (that corresponding to LWS in this case) and their natural logarithm was then calculated. Thus, three log-contrasts were calculated to break the unit-sum of the four standardized cone catches (Hadfield and Owens 2006). A principal components analysis (PCA) using the raw data was performed on the three log-contrast colour variables. Factor loadings for the first axis (PC1) were high and negative in all patches, indicating that it is determined by the three first cone catches vs LWS. Thus, high PC1 scores indicate relatively higher reflectance at long wavelengths as compared to short/medium wavelengths (Table 1; Galván 2010). The second axis (PC2) was always determined by MWS vs LWS cone catches (Galván 2010), and thus high PC2 scores indicate relatively higher reflection at long wavelengths as compared to medium wavelengths (Table 1; Galván 2010).

Table 1. Factor loadings obtained from the principal components analysis (PCA) performed on the different log-contrasts (UVS, SWS and MWS) of three plumage patches in great tits. Taken from Galván (2010).

	PC1	PC2
Yellow breast Colour	UV/blue/green vs red	Green vs red
Eigenvalue	2.73	0.25
% variance	90.88	8.30
UVS log-contrast	−0.97	0.21
SWS log-contrast	−0.98	0.18
MWS log-contrast	−0.91	−0.42
Black breast stripe Colour	UV/blue/green vs red	Green vs red
Eigenvalue	2.69	0.28
% variance	89.76	9.23
UVS log-contrast	−0.94	0.31
SWS log-contrast	−0.98	0.08
MWS log-contrast	−0.91	−0.41
Green back Colour	UV/blue/green vs red	Green vs red
Eigenvalue	2.72	0.26
% variance	90.72	8.77
UVS log-contrast	−0.99	0.10
SWS log-contrast	−0.95	0.28
MWS log-contrast	−0.91	−0.41

As achromatic variation in birds is thought to not be perceived through the four single cones, but by double cones (Osorio et al. 1999), the double cone quantum catches were calculated following the same procedure for the four single cones. The double cone quantum catch was used as a 'brightness index'.

Feather microstructure

Feathers plucked from birds were analysed with a FEI QUANTA 200 scanning electron microscope (SEM) (FEI, Eindhoven, the Netherlands) operating in low vacuum mode and 25 kV and using a wavelength dispersive X-ray detector (WDS). Only the uppermost feather collected was analysed per bird. The feathers were cut perpendicularly to the rachis with a scalpel at the point of insertion of the tenth distal-most barb in the rachis. The samples were mounted horizontally on stainless-steel pegs by using double sided graphite tape before being transferred to the SEM chamber. A micrograph was obtained from these samples, and the pairs of distal and proximal barbules per 0.5 mm length of barb, from the point of insertion in the rachis, in three barbs chosen at random were counted (Fig. 1b). The mean of these counts was used. Feather samples were also mounted on an upright position (Fig. 1c) to obtain a micrograph of the cross-section of the 12th distal-most barb by positioning the barb axis perpendicular to the SEM detector's plane (Fig. 1d). The surface areas covered by the keratin cortex and the pith of the barb (Fig. 1d) were measured. Image analyses were made with Photoshop CS.

Statistical analyses

Since feather microstructure variables were highly correlated among them, the relationships between colour expression (response) variables and predictor variables (number of barbules, barb cortex size, barb pith size and ratio cortex:pith size) were analysed with partial least squares regressions (hereafter PLSR; Carrascal et al. 2009). This statistical tool is an extension of multiple regression analysis where associations are established with factors extracted from predictor variables that maximise the explained variance in the dependent variable. These factors are defined as a linear combination of independent variables, so the original multidimensionality is reduced to a lower number of orthogonal factors to detect structure in the relationships between predictor variables and between these factors and the response variable. The extracted factors account for successively lower proportions of original variance (Carrascal et al. 2009). The relative contribution of each variable to the derived PLSR components was calculated by means of the square of the predictor weights. The significance of the PLSR components was tested by cross-validation.

The models were controlled for the effect of body condition, as this variable, whose values at moult (i.e. end of summer) and in winter are correlated in great tits (Gosler and Harper 2000), is related to plumage colour traits in great tits (Galván 2010) and may be also correlated with elements of feather microstructure. Thus, body condition

was added as a covariate. The results of PLSR models were corroborated by Pearson correlation tests between the response variables and the most important predictors (i.e. those accounting for $>40\%$ of the variance explained by the PLSR axes). The presence of outliers was determined on the basis of Cook's distances >2 and leverages $>2p/n$, where p is the number of parameters in the model and n is the sample size. These analyses were made with Statistica 8.0 (StatSoft).

Results

The four PLSR models for the black breast stripe resulted in one significant component each. PC1 scores were positively related to barbule density and negatively related to barb cortex:pith size ratio (Table 2), indicating that relative smaller barb cortex and higher barbule density create longer reflectance (relative to short reflectance) in the breast stripe of birds. PC2 scores were negatively related to barbule density, barb cortex size and barb cortex:pith size ratio (Table 2), indicating that absolute and relative smaller barb cortex size and lower barbule density create longer reflectance (relative to medium reflectance). The brightness index was negatively related to barbule density, barb cortex size and barb cortex:pith size ratio and positively related to barb pith size (Table 2), indicating that absolute and relative larger barb cortex size, absolute and relative smaller barb pith size and greater barbule density create darker breast stripes. These results did not change when females were excluded from the analyses, except that the effect of barbule density on PC2 scores was weaker (Table 2).

Breast stripe size was negatively related to barb cortex and pith sizes and barbule density, and positively related to barb cortex:pith size ratio (Table 2). Again, the same was obtained when females were excluded from the analyses (Table 2). These results were corroborated by correlation tests for the most important predictors (Fig. 2a–c). A point with a high brightness index (Fig. 1a) was discarded from being an outlier because its Cook's distance and leverage were 0.57 and 0.15, respectively ($2p/n = 0.20$).

PLSR models for the other plumage patches did not result in any significant component (Table 2), except the PLSR model for PC1 scores of the green back patch. The main predictor of this model was barbule density (predictor weight $= 0.64$), but when an outlier was removed (leverage $= 0.18$, $2p/n = 0.17$; Fig. 2d), the PLSR component was no longer significant.

Discussion

The components of feather microstructure considered here predicted the degree of expression of the melanin-based plumage, but not of carotenoid-based patches. This is the first time that the structure of feathers is related to the colour expression of purely melanin-based (i.e. non-structural) plumage and to the size of a plumage patch. Great tits of both sexes with larger breast stripes get better access to resources and higher mating success, so this trait acts as a honest signal of quality (Järvi and Bakken 1984, Norris 1990, Carrascal et al. 1998, Quesada and Senar 2007). The same applies to other species, where melanin-based traits have evolved as signals of individual quality (Jawor and Breitwisch 2003, Hoi and Griggio 2008, Griggio and Hoi 2010). The mechanisms maintaining the honesty of melanin-based traits are currently debated, however (Galván and Alonso-Alvarez 2008, 2009).

It has been shown that a key intracellular antioxidant (i.e. glutathione, GSH) is involved in the expression of melanin-based traits, so that low values of GSH are needed for the production of large amounts of eumelanin (i.e. the melanin type that generates black traits like the breast stripe of great tits). Thus, the expression of large melanin-based traits could be limited to individuals presenting a high antioxidant capacity (Galván and Alonso-Alvarez 2008, 2009). The results of the present study suggests that the costs of producing melanin-based traits may be even more complex if elements of feather microstructure are considered. In particular, here it is shown that low barbule density is associated with large breast stripes, suggesting a trade-off between structural integrity of feathers and large stripes, similarly to the suggested trade-offs between structural integrity and structural colour expression (Shawkey et al. 2005) and body growth (Butler et al. 2008). A high barbule density may increase the structural integrity of feathers because this would provide a greater number of points of attachment between barbules of different barbs. However, if the synthesis of melanin is costly in antioxidant terms (Galván and Alonso-Alvarez 2008, 2009) and the synthesis of the structural component of feathers and barbules (i.e. keratin) is also limited by the availability of proteins (Peters et al. 2007), generating a breast stripe that is both large (i.e. containing large amounts of melanin) and presents a high structural integrity (i.e. containing a high density of barbules) may be particularly costly. This may explain the negative association between barbule density and breast stripe size found here.

By contrast, high barbule density was related to dark stripes, probably because more barbules contain larger amounts of melanin that absorb more light, as both barbs and barbules are pigmented in great tits. Although the colour of the breast stripe of great tits has never been investigated in a signalling context, darkness is positively related to the melanin content and is under sexual selection in melanin-based signals (Galván and Møller 2009). Thus, dark breast stripes are potentially indicative of high quality in addition to large stripes. The positive relationship between stripe darkness and barbule density suggests that size and darkness of this trait may be controlled by different mechanisms. Together with the fact that dark stripes were also associated with great barb cortexes and small piths, this also suggests that part of variation in the darkness of melanin-based plumage also depends on the microstructure of feathers and not only on melanin content. Relative long reflectance of the breast stripe was also affected by barbule density and barb cortex and pith sizes. However, these results should be taken with caution due to the correlational nature of the study, and the possibility that the findings presented here are a consequence of a

Table 2. Predictor weights of the four PLSR analyses explaining the relationship between colour parameters and size of the black breast stripe of great tits (response variables) and microstructural components of feathers and body condition (predictor variables). Predictor weights that retain >5% of the information content of the PLSR axis are shown in bold. Only PLSR components for the black breast stripe and the PLSR component for PC1 of the green back were significant by cross-validation. Results including both males and females and males only are shown.

Black breast stripe Predictor variable	PC1 score	PC1 score (males only)	PC2 score	PC2 score (males only)	Brightness index	Brightness index (males only)	Breast stripe width (mm)	Breast stripe width (mm) (males only)
No. barbules 0.5 mm^{-1} barb	**0.70**	**0.48**	**−0.26**	−0.01	**−0.76**	**−0.72**	**−0.68**	**−0.63**
Barb cortex size (μm^2)	0.04	−0.13	**−0.70**	**−0.67**	**−0.24**	**−0.24**	**−0.40**	**−0.37**
Barb pith size (μm^2)	0.08	−0.02	0.01	0.10	**0.25**	**0.34**	**−0.48**	**−0.51**
Barb cortex:pith ratio	**−0.34**	**−0.37**	**−0.62**	**−0.70**	**−0.26**	**−0.32**	**0.38**	**0.45**
Body condition (g mm^{-3})	**−0.62**	**−0.78**	**−0.23**	−0.20	−0.49	−0.45	–	–
% variance accounted for	26.66	24.76	22.63	29.90	26.52	23.1	15.93	12.11
Yellow breast Predictor variable	PC1 score	PC1 score (males only)	PC2 score	PC2 score (males only)	Brightness index	Brightness index (males only)	–	–
No. barbules 0.5 mm^{-1} barb	**0.71**	**0.68**	**0.57**	**0.71**	**−0.34**	−0.08	–	–
Barb cortex size (μm^2)	**0.26**	**0.25**	**−0.30**	−0.16	−0.02	**−0.25**	–	–
Barb pith size (μm^2)	0.05	**−0.36**	0.17	0.21	0.13	0.00	–	–
Barb cortex:pith ratio	**−0.26**	−0.02	**−0.41**	**−0.30**	**−0.44**	**−0.56**	–	–
Body condition (g mm^{-3})	**0.59**	**0.58**	**−0.62**	**−0.58**	**−0.82**	**−0.78**	–	–
% variance accounted for	1.70	0.77	12.87	12.56	13.10	14.38	–	–
Green back Predictor variable	PC1 score	PC1 score (males only)	PC2 score	PC2 score (males only)	Brightness index	Brightness index (males only)	–	–
No. barbules 0.5 mm^{-1} barb	**0.64**	**0.32**	−0.16	**0.92**	**0.44**	**0.47**	–	–
Barb cortex size (μm^2)	**−0.56**	**−0.76**	**0.51**	−0.02	**−0.49**	**−0.51**	–	–
Barb pith size (μm^2)	**−0.51**	**−0.49**	**0.82**	**0.35**	**0.27**	0.20	–	–
Barb cortex:pith ratio	−0.01	**−0.21**	−0.19	−0.14	**−0.68**	**−0.58**	–	–
Body condition (g mm^{-3})	0.08	−0.16	−0.06	0.05	0.16	**0.37**	–	–
% variance accounted for	8.01	4.47	0.59	0.00	7.82	12.54	–	–

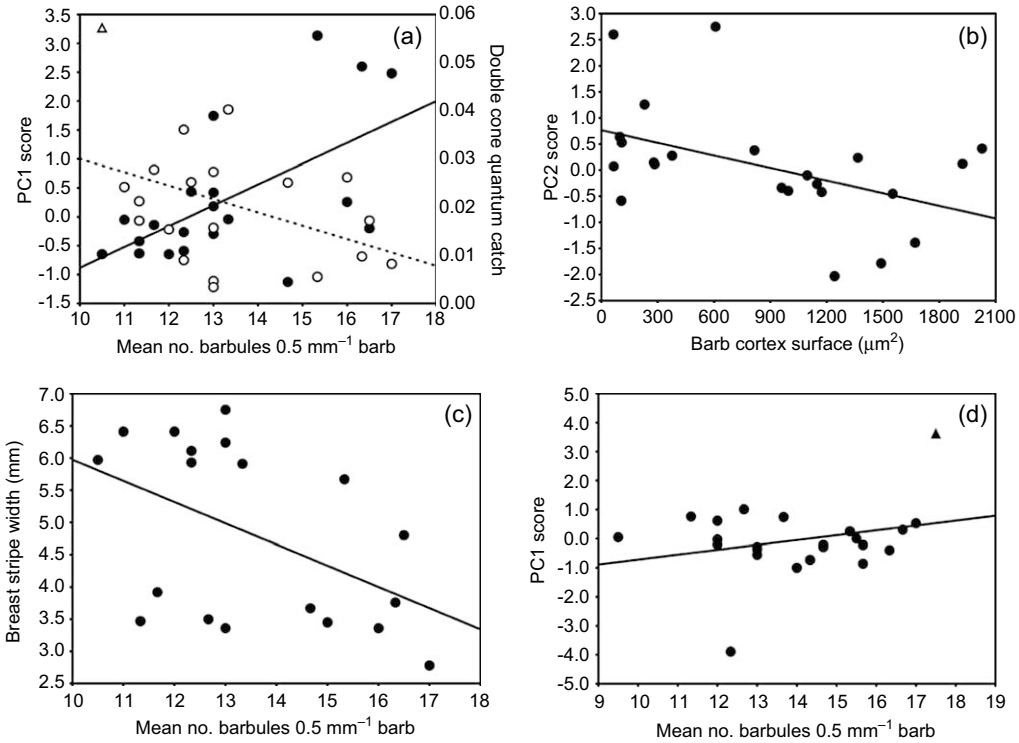

Figure 2. Relationships between colour and size of plumage patches and the most important feather microstructure predictors (i.e. those accounting for >40% of the variance explained by the PLSR axes) in great tits. (a) Relationship between barbule density and PC1 score (black line, solid symbols; r = 0.59, n = 20, p = 0.006) and double cone quantum catch ('brightness index', dashed line, hollow symbols; r = −0.41, n = 20, p = 0.072) for the black breast stripe of great tits. (b) Relationship between barb cortex size and PC2 score for the black breast stripe (r = −0.46, n = 23, p = 0.026). (c) Relationship between barbule density and breast stripe size (r = −0.48, n = 19, p = 0.038). (d) Relationship between barbule density and PC1 score for the green back patch (r = 0.27, n = 23, p = 0.209). High PC1 scores indicate relatively more reflectance at long wavelengths as compared to short/medium wavelengths, while high PC2 scores indicate relatively more reflection at long wavelengths as compared to medium wavelengths. Triangles represent potential outliers. The lines are the regression lines.

correlation between melanin levels in feathers and elements of feather microstructure, although unlikely, should not be discarded. Future studies that consider the amount of melanin deposited in feathers should corroborate these conclusions.

Feather microstructure did not predict the colour expression of the carotenoid-based plumage patches considered here, suggesting that carotenoid-based colour may depend more on the amount or composition of the carotenoids in feathers than on the development of feather microstructural elements. The effect of feather microstructure on the expression of carotenoid-based colour (Shawkey and Hill 2005) may thus be exerted by different microstructural feather elements than in melanin-based plumage colour. Given the debate on whether carotenoid- and melanin-based colour signals have or not similar information contents (Griffith et al. 2006), these results suggest that the role of feather microstructure, and not only of pigments, should be considered when investigating how the mechanisms of both types of plumage colouration explain their function and evolution.

In sum, these findings indicate that variation in the microstructure of feathers can be perceived by birds by assessing the expression of plumage colour. This is compelling for understanding the evolution of melanin-based signals in birds.

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