Relationships between territory quality and carotenoid-based plumage colour, cell-mediated immune response, and body mass in Great Tit *Parus major* nestlings

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Abstract. Plumage colour is classified as pigmentary or structural, depending on whether it is caused by pigments or by feather microstructure. However, recent findings indicate that carotenoid-based plumage colouration also reflects at UV-blue wavelengths and that the underlying structure is related to the reflectance properties of the yellow feathers. Thus, yellow plumage is based on interactions between structural and pigmentary components. This study investigated the relationships among the vegetation structure of breeding territories, both components of plumage colour, T-cell-mediated immune response and body mass of nestling Great Tits *Parus major*. By using a model of avian visual perception, we found that, while plumage yellowness was associated with mature vegetation, plumage brightness and UV-blue reflectance were related to immature habitats in territories. We noted considerable variability in the development of carotenoid-based colour components under different environmental conditions, as plumage yellowness, but not brightness or UV-blue reflectance, depends on the availability of carotenoids, which is assumed to be high in mature territories with high food abundance. Territorial features denoting mature territories were also related to high body mass and immune response in nestlings, but none of the colour components were related to these variables of the vegetation structure, suggesting that habitat quality is related to nestling body mass and immune response through mechanisms different from those through which it is related to colour.

Key words: carotenoids, phenotypic plasticity, plumage colour, T-cell immune response, territory quality

INTRODUCTION

Plumage colour is based on either pigments (mainly carotenoids and melanins) or on feather microstructure (Brush 1978). The two types of pigments differ in the way birds acquire them, as, in contrast to melanins, carotenoids can not be synthesized de novo by vertebrates. Carotenoids have to be ingested with food, and are involved in important physiological functions because, among other things, they have immunostimulant and antioxidant properties, which makes them indispensable components of the vertebrates' diet (Lozano 1994, Olson & Owens 1998, Møller et al. 2000).

Because the availability of food containing carotenoids in the environment is related to the expression of plumage colour, plumage colour could reflect habitat quality (Slagsvold & Lifjeld 1985, Eeva et al. 1998, Wolfenbarger 1999, Hõrak et al. 2000, Arriero & Fargallo 2006, Ferns & Hinsley 2008). Although some studies have compared plumage colouration in habitats in different stages of degradation (Slagsvold & Lifjeld 1985, Eeva et al. 1998, Hõrak et al. 2000, Isaksson & Andersson 2008) or considered indirect measures of habitat quality such as territory or forest patch size (Pryke et al. 2001, Ferns & Hinsley 2008), the influence of particular structures of breeding territories on carotenoid-based colour is poorly known (but see

Hill 1988, Wolfenbarger 1999, Arriero & Fargallo 2006). Arriero & Fargallo (2006) have found that nestling carotenoid-based plumage colour is more affected by territory features than by parental colouration in Blue Tits *Cyanistes caeruleus*. Furthermore, a recent experimental study indicates that variation in plumage colour of Great Tit *Parus major* nestlings is more likely to be determined by the quality of the food provided by the parents (which depends on territory quality) than by genetic effects (Isaksson et al. 2006). Indeed, there is a great environmental-based variation in the plumage colour of Great Tits among individuals within populations (Postma & Gienapp 2009).

Recent studies demonstrate that the amount of carotenoids in feathers is related to plumage hue and saturation, but not to brightness (Saks et al. 2003, Shawkey et al. 2006). The total amount of light (i.e. brightness) reflected by carotenoid-based plumage is, at least in part, determined by a white structure underlying the pigmented part of feathers (Shawkey & Hill 2005). This means that the brightness of carotenoid-based plumage colour reflects structural properties of feathers. Therefore, similar to purely structural colours (e.g., Keyser & Hill 1999, 2000, Siefferman & Hill 2005), the brightness of carotenoid-based plumage may be related to physiological condition during feather growth because it could reflect the capacity to generate nanostructural components of feathers, though the mechanisms that generate the underlying white reflectance are unknown (Shawkey & Hill 2005). Conversely, its chromaticity (i.e. hue and saturation) reflects the amount of carotenoids (Saks et al. 2003, Shawkey et al. 2006) and thus should be related to changes in carotenoid availability in the environment (e.g. caterpillars in the case of Great Tits; Partali et al. 1987). Some authors have suggested that different ornaments, such as different plumage colours (e.g. Senar et al. 2003), might signal different aspects of individual quality (Møller & Pomiankowski 1993). However, the potential of different colour components in the same colour patch to have different signaling roles has rarely been considered (see however Badyaev et al. 2001, Grether et al. 2004, Senar et al. 2008). Instead, the three main dimensions of colour perception have been often treated as redundant elements with respect to their signaling function, and only Siefferman & Hill (2003; for a structural colour) and Stein & Uy (2006) and Senar et al. (2008; for a carotenoid-based colour) have recently discussed the issue.

Since carotenoids are immunostimulant, any effect of habitat on carotenoid-based color may be correlated with the immune response of birds at the same time. The acquisition of carotenoids in nestlings is even more important than during other developmental stages of birds (Hõrak et al. 2000), as the immune system of altricial nestlings is poorly developed (Klasing & Leshchinsky 1999) and they are unable to escape from parasites, while the rapid growth they experience results in the production of abundant free radicals (Surai et al. 1999). Indeed, higher levels of carotenoids in both the plasma and the integument of birds are related to stronger cellular and humoral immune responses or to higher lymphocyte levels (e.g. Bortolotti et al. 2000, Alonso-Alvarez et al. 2004, Peters et al. 2004, but see Biard et al. 2006), and thus there are also positive relationships between the expression of carotenoid-based colours and the intensity of immune responses (e.g. Faivre et al. 2003, Alonso-Alvarez et al. 2004, Peters et al. 2004). Again, the effect of characteristics of breeding territories on the T-cell proliferative response of nestlings through the availability of carotenoids has been overlooked, despite the immune response being largely influenced by environmental conditions (Sorci et al. 1997, Tella et al. 2000, Jovani et al. 2004, Moreno et al. 2005).

On the other hand, plumage colouration may be conditioned by nestling body mass because larger clutches can result in nestlings of poorer quality (Hõrak et al. 2000, Tschirren et al. 2003), and nestling mass is negatively correlated with clutch size (Sanz & Tinbergen 1999). High quality habitats are known to be positively correlated with the body mass of nestlings (Verhulst et al. 1997, Sánchez et al. 2007), which is a predictor of autumn fledgling survival (Tinbergen & Boerlijst 1990). Therefore, habitat effects on plumage colour may be accompanied by effects on nestling body mass. However, the amount of carotenoids available to nestlings does not seem to be related to their body mass (Hõrak et al. 2000, Tschirren et al. 2005), which suggests that habitat quality may affect the plumage colouration and mass of chicks through different mechanisms (Hõrak et al. 2000).

The aim of the present study is to determine whether characteristics of breeding territories are related to different colour components like brightness or ultraviolet (UV) chroma of the yellow plumage of nestling Great Tits. If characteristics of the breeding territories are related to the expression of carotenoid-based colour (Arriero & Fargallo 2006) but brightness and chromaticity are

generated by different mechanisms (Saks et al. 2003, Shawkey & Hill 2005), then different territory features may be related to these colour components. We used current models of avian colour vision (Vorobyev et al. 1998, Endler & Mielke 2005) in order to calculate plumage colour components that are perceived by birds and thus have the potential of being significant from a signaling perspective in parent-offspring communication (Galván et al. 2008) or in the post-fledging period.

Additionally, we expected that, if high carotenoid availability enhances the cellular immune response of nestlings (Blount et al. 2003, Alonso-Alvarez et al. 2004), those Great Tits reared in territories with characteristics associated with high food availability and thus potentially high carotenoid availability should mount stronger responses. Finally, we considered the body mass of nestlings in order to determine whether habitat quality differentially affects this variable and plumage colour components as suggested by previous studies (Hōrak et al. 2000, Tschirren et al. 2005).

MATERIALS AND METHODS

The study was carried out in May–June 2005 in a deciduous forest of Pyrennean Oak *Quercus pyrenaica* in Miraflores de la Sierra, Sierra de Guadarrama, central Spain (40°49′ N, 03°46′ W, 1352 m a.s.l.). 125 wooden nest-boxes of a standard size for tits had been placed in this forest and cleaned before the start of the breeding season. Frequent checks of nest-boxes allowed us to collect data on dates of clutch initiation and clutch size for all breeding pairs. Nestlings were ringed on day 14 after hatching (hatching date = day 0) and weighed with a portable electronic balance to the nearest 0.1 g.

The study forest is mainly monospecific in tree composition, having sparse Narrow-leafed Ashes *Fraxinus angustifolia* along streams. However, its vegetation structure is highly heterogeneous, ranging from cleared forest patches used for cattle grazing to forest patches dominated by highly dense vegetation at both understorey and canopy layers.

Reflectance spectrometry

On day 14–15 after hatching, the yellow colour of the breast was analysed from nestlings of 15 pairs (105 nestlings in total). Data were collected in the field using an Ocean Optics USB2000 spec-trophotometer (range 250–800 nm) with ultraviolet (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400 micrometer fiber-optic probe (Dunedin, Florida, USA). The fiber optic probe both provided illumination and obtained light reflected from the sample, and had a reading area of ~1 mm². 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, Dunedin, Florida, USA), and reference measurements were frequently made. The spectral curves were generated by using OOIBase software. Similar to Mennill et al. (2003), three readings were obtained on different points of the upper part of the right side of the yellow breast of Great Tit nestlings, moving the probe by at least 5 mm before taking each new reading, but always following the same order (from upper to lower breast).

Visual modelling

Colour vision in diurnal birds depends on four types of single retinal cone cells, which are sensitive to very short (VS, UV), short (SWS, blue), medium (MWS, green) and long (LWS, red) wavelengths, respectively (Cuthill 2006). 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 & Owens 2006) implemented in R (R Development Core Team 2004), the four quantum cone catches 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:

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

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 (μmol*m^{-2}*s^{-1}*nm^{-1}) and $O(\lambda)$ is the transmittance spectrum of the ocular media.

The cone catches were normalized using the von Kreis algorithm with standard forest shade illumination, as this irradiance spectrum is the most appropriate for our case (see habitat description above), and an achromatic adapting background (Hadfield & Owens 2006). We used the

ocular media transmittance of a species closely related to the Great Tit (i.e. the Blue Tit; 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). The natural logarithm was then taken in order to calculate three log-contrasts that break the unit-sum of the four standardized cone catches (Hadfield & Owens 2006). In addition, these log-contrasts permit us to consider an opponency model of colour vision where the exact opponency mechanisms are unspecified, as perception of chromatic variation occurs through a physiological mechanism that contrasts the relative outputs of the different cone types (Endler & Mielke 2005, Hadfield & Owens 2006).

Following Hadfield & Owens (2006), a principal components analysis (PCA) was performed on the three log-contrast colour variables in order to treat them as a multivariate response. In the case of the breast plumage colour, the eigen decomposition showed a first axis that explained 86% of phenotypic variation, while the other tree axes explained 12% and 2% of variance, respectively (Table 1). Factor loadings for the first axis (PC1) were high and negative, indicating that it is determined by the three first cone cathes vs. LWS (Table 1). Thus, high PC1 scores indicate relatively more reflectance at long wavelengths as compared to short/medium wavelengths. The second axis (PC2) is determined by MWS vs. LWS cone catches (Table 1), and thus high PC2 scores indicate relatively more reflection at long wavelengths as compared to medium wavelengths. The variance explained by the third axis was very low (2%), and hence PC3 was not considered in the analyses.

As brightness (i.e. achromatic) variation in birds in thought to not be perceived through the four single cones, but by double cones (Campenhausen & Kirschfeld 1998, Osorio et al. 1999), we calculated the double cone quantum catches from data on double cone spectral sensitivity of Blue Tits following the same procedure for the four single cones. The double cone quantum catch was used as a 'brightness index'.

Habitat structure

Structural features of 15 breeding territories were visually estimated by a single trained observer (L.D.) shortly after the breeding season within a 25 m radius circular plot surrounding each nest-box, since the majority of movements of breeding Great Tits take place inside this area (Naef-Daenzer 1994). The vegetation variables estimated were covers of rocks, leaves, and herbaceous and shrub layers (i.e. *Quercus pyrenaica*, *Cytisus scoparius* and *Genista florida* covers), the average height of shrubs and trees, and the number of trees according to three categories of diameter at breast height (DBH): thin (DBH: 5–9 cm), medium-sized (DBH: 10–29 cm) and thick (DBH: > 30 cm).

Vegetation features included in the models were previously selected on the basis of results of multiple regression models showing the relationship between food availability (i.e., potential indicator of carotenoid availability) and vegetation structure and floristic composition in the study area during the survey (i.e., peak of caterpillar abundance; results not shown). In the study area, the understorey comprises several shrub species, mainly young oaks, *Genista florida* and *Cytisus scoparius*. Based on these field observations, the two latter species do not provide a suitable substrate to allow the development of caterpillars (i.e., small leaves compared to oaks). However, both *G. florida* and oaks are positively related to the developmental stage of the understorey (i.e., to height and total cover of shrubs). Therefore, in the analyses of nestling colour we also differentiated between cover of young oaks, *Cytisus scoparius*, and *Genista florida* in order to specify the influence of potentially favourable (young oaks) and unfavourable (*C. scoparius* and *G. florida*) developmental substrates of caterpillars in the understorey, which is one of the preferred feeding substrates of Great Tits. We were thus able to characterise habitats as having more or less of the kinds of vegetation preferred by Great Tits, with regenerating forest having more of the preferred vegetation type.

Table 1. Factor loadings obtained from the principal components analysis (PCA) performed on the different log-contrasts (UVS, SWS and MWS, i.e. log-contrasts of the catches of the retinal cones sensitive to very short, short and medium wavelengths, respectively, against that of the cone sensitive to long wavelengths) of the breast plumage of Great Tit nestlings.

Breast patch Colour	PC1 UV/blue/ green vs. red	PC2 Green vs. red	PC3 -
Eigenvalue	2.58	0.36	0.06
% variance	85.99	12.05	1.96
UVS log-contrast	-0.95	0.26	0.16
SWS log-contrast	-0.96	0.19	-0.18
MWS log-contrast	-0.86	-0.50	0.02

Immune response

The protocol described by Smits et al. (1999) was used to evaluate the T-cell-mediated immune response of nestlings when they were 14–15 days-old. This test measures the proliferative response of circulating T-lymphocytes to the intradermal injection of a mitogen (phytohaemagglutinin; PHA). We injected 0.2 mg of PHA (Sigma, StLouis, MO, USA) in 0.04 ml of phosphate-buffered saline (PBS) subcutaneously in the right wing-web after measuring the web thickness at the point of injection with a micrometer (Mitutoyo 547-301, Tokyo, Japan) to the nearest 0.001 mm. A new measurement of wing-web thickness was taken 24 h later, and the T-cell-mediated immune response was calculated as the difference between initial and final measurements. Stronger T-cell responses are thus indicated by larger positive changes in the wing-web thickness. Three replicate measurements of the wing-web were taken each time, and the measures' repeatability was estimated by means of the intraclass correlation coefficient (Lessells & Boag 1987). Wing-web thickness measures were highly repeatable, both for measures taken before the injection of the mitogen ($r = 0.923$, $p < 0.0001$), and for those taken 24 h later ($r = 0.986$, $p < 0.0001$).

Statistical analyses

The relationships between nestling plumage colour variables, body mass and PHA response (response variables) and habitat characteristics (predictor variables) were analysed by means of partial least squares regressions (hereafter PLSR; e.g., Frank & Friedman 1993, Swold et al. 2001, Tobias 2003), using the breeding territory as the sample unit and thus mean values of those variables per nest. Possible effects of brood size were controlled for by introducing the total number of nestlings in each nest on day 15 in the analyses. This statistical method is an extension of the multiple regression analysis where the effects of linear combinations of several predictors on a response variable can be analysed. Associations are established with factors extracted from predictor variables that maximize 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. The relative contribution of each variable to the derived factors was calculated by means of the square of predictor weights. When multiple response variables are used, PLSR creates a synthetic response variable from the linear combination of the original response variables. Results obtained with PLSR are similar to those from conventional multiple regression techniques, but it is extremely robust to the effects of sample size and degree of correlation between predictor variables, which makes PLSR especially useful when sample size is low and in cases of severe multicollinearity (Carrascal et al. 2009). Additionally, PLSR can be applied when the number of predictor variables is higher than the number of cases, a situation where other regression methods fail (Frank & Friedman 1993, Swold et al. 2001, Tobias 2003, Carrascal et al. 2009).

Possible relationships between plumage colour variables, body mass and T-cell immune response were explored by using multiple regression analyses in which the different axes segregated by the PCA performed for the log-contrasts and the brightness index were considered as dependent variables on the others (body mass and immune response). Explorations of the residuals of the analyses confirmed that they were normally distributed.

RESULTS

Relationship between body mass, immune response and carotenoid-based colour

The PCA axes calculated for breast colour and brightness index were not related to either body mass or immune response (multiple regression; PC1: PHA response: $\beta_9 = -0.13$, $p = 0.688$; body mass: $\beta_9 = -0.33$, $p = 0.323$; PC2: PHA response: $\beta_9 = 0.20$, $p = 0.579$; body mass: $\beta_9 = 0.00$, $p = 0.997$; Brightness index: PHA response: $\beta_9 = -0.42$, $p = 0.220$; body mass: $\beta_9 = 0.20$, $p = 0.434$).

Relationship between territory features and carotenoid-based colour

Results of the PLSR relating PC scores obtained for breast plumage colour to habitat structure and brood size are summarized in Table 2. A single PLSR axis was extracted, explaining 43.8% of inter-nest variability in plumage colour. This axis was negatively related to PC1 and positively to PC2 and brightness index, meaning that the higher values of nestling plumage brightness

Table 2. Results of the partial least squares regression (PLSR) analysis explaining the relationship between nestling colour components (brightness index, PC1 and PC2; response variables) of the breast plumage patch and vegetation structure of the breeding territories of Great Tits (predictor variables). Predictor weights represent the contribution of each territory characteristic to the PLSR axis. Response weights correspond to the contribution of either brightness index, PC1, PC2 or PC3 colour component to the synthetic response variable built in each PLSR. The relationship between the synthetic response variable of breast plumage colour (linear combination of PCs) and the PLSR axis is also provided. Predictor weights explaining more than 10% of the total variance (43.8%) are underlined. See Materials and Methods for further explanations.

Predictor variables	Predictor weights
Brood size	-0.04
Rock cover (%)	0.20
Leaf cover (%)	-0.06
Herbs cover (%)	<u>-0.33</u>
Quercus pyrenaica cover (%)	-0.06
Citysus scoparius cover (%)	0.05
Genista florida cover (%)	<u>0.49</u>
Shrub height (m)	0.04
Tree height (m)	0.14
No. thin trunks	<u>0.43</u>
No. medium-sized trunks	<u>0.43</u>
No. thick trunks	<u>-0.46</u>
Response variables	**Response weights**
Brightness index	0.76
PC1	-0.62
PC2	0.21
Relationship between the synthetic response variable and PLSR axis	
r Spearman	0.871
p value	< 0.0001

and long-wavelength in relation to medium-wavelength reflectance were related to habitats with high densities of oaks of thin and medium-sized trunks and abundant *Genista florida* in the shrub layer. Conversely, the highest values of long-wavelength reflectance as compared to the rest of spectral regions (mainly short wave-lenghts) were encountered in areas of thick oaks and abundant herbs on the ground. Brood size was not related to breast colour (Table 2).

It is noteworthy that the response weights obtained from the PLSR analyses were of different signs for the first axis segregated by the PCA performed on plumage colour (PC1) and for the brightness index (see Table 2). Since positive values of breast PC1 denote higher relative reflectance at long wavelengths in relation to short-wavelength reflectance (see Table 1), that means that the structural features of breeding territories explaining breast colour characteristics

of nestlings were the same for plumage brightness and UV-blue reflectance, whereas different features were related to those plumage spectra showing high yellowness.

Relationship between territory features and body mass

A third PLSR analysis was conducted to examine the relationship between nestling body mass and habitat features and brood size (Table 3). Two PLSR axes positively related to nestling body mass (46.9% of total inter-nest variations) were obtained. The first PLSR axis (PLSR1: 24.6% of total variance) was not significantly related to body mass, but a tendency suggesting that nestling mass was higher in forest patches with tall oaks, high rock cover and shrub layers with abundant *Citysus scoparius* but with few resprouting oaks was observed (see Table 3). The second PLSR axis (PLSR2: 22.4% of total variance) was significantly related to body mass, and reinforced the tendency observed in PLSR1 that the heaviest chicks were encountered in territories with tall oaks producing abundant litter and few young oaks in both shrub and canopy layers. Nestling body mass was independent of brood size (Table 3).

Table 3. Results of the partial least square regression (PLSR) analysis relating vegetation structure of breeding territories of Great Tits to nestling body mass. Predictor weights of the variables comprising each PLSR axis, as well as response weights explaining the relationships between body mass and each PLSR axis are shown. Correlations between response (body mass) and predictor variables (PLSR axes) are also provided. Predictor weights explaining more than 10% of the total variance (46.9%) are underlined.

	Axis 1	Axis 2
Predictor variables	Predictor weights	
Brood size	0.08	0.27
Rock cover (%)	<u>0.36</u>	0.2
Leaf cover (%)	0.09	<u>0.39</u>
Herbs cover (%)	-0.21	-0.28
Quercus pyrenaica cover (%)	<u>-0.55</u>	<u>-0.34</u>
Citysus scoparius cover (%)	<u>0.42</u>	0.15
Genista florida cover (%)	0.3	-0.08
Shrub height (m)	-0.03	-0.02
Tree height (m)	<u>0.41</u>	<u>0.6</u>
No. thin trunks	0.2	-0.04
No. medium-sized trunks	-0.11	<u>-0.39</u>
No. thick trunks	-0.17	-0.06
Response variable	Response weights	
Body mass	0.49	0.47
Relationship between the response variable and PLSR axes		
r Spearman	0.411	0.589
p value	0.128	0.021

Relationship between territory features and T-cell-mediated immune response

Finally, a fourth PLSR was used to describe the relationships between the immune response of nestlings to PHA to habitat characteristics and brood size (Table 4). The two first axes explained 73.3% of total variance, and both were positively related to the immune response of chicks. The first one (PLSR1: 49.8% of total variance) showed that the immune response of nestlings was correlated positively with brood size, but negatively with oak maturity (medium-sized and thick trunks). The second PLSR axis (PLSR2: 23.6% of total variance), strengthened the first one, showing how the immune response of nestlings was negatively related to the development of trees, while positively to the abundance of *Genista florida* in the shrub layer.

DISCUSSION

Relationship between territory quality and plumage colour

Great Tit nestlings showing high values of plumage brightness and short- and medium-wavelenght reflectance in relation to long-wave-

Table 4. Results of the partial least square regression (PLSR) analysis for Great Tit nestling T-cell immune response. Predictor weights of the variables comprising each PLSR axis, as well as response weights and correlation explaining the relationships between the immune response and each PLSR axis are provided. Predictor weights explaining more than 10% of the total variance (73.3%) are underlined.

	Axis 1	Axis 2
Predictor variables	Predictor weights	
Brood size	0.56	0.2
Rock cover (%)	-0.22	0.05
Leaf cover (%)	0.02	-0.39
Herbs cover (%)	0.25	0.14
Quercus pyrenaica cover (%)	0.04	-0.28
Citysus scoparius cover (%)	-0.27	0.12
Genista florida cover (%)	-0.04	0.45
Shrub height (m)	-0.09	-0.24
Tree height (m)	0.16	-0.02
No. thin trunks	-0.28	-0.28
No. medium-sized trunks	-0.31	-0.32
No. thick trunks	-0.54	-0.51
Response variable	Response weights	
T-cell immune response	0.70	0.48
Relationship between the response variable and PLS axes		
r Spearman	0.790	0.650
p value	0.002	0.022

length were reared in territories with high densities of thin and medium-sized oaks and *Genista florida* shrubs, which reflects forest patches of young trees and sparse shrub layer (leaves of *Genista* shrubs are too thin to allow caterpillar development; pers. obs.). This also applied to long-/medium-wavelength and UV/blue reflectance ratios. Conversely, the highest values of plumage yellowness (i.e., reflectance at long wavelengths relative to reflectance at other spectral regions) were associated with territories with abundant thick oaks accompanied by high herb cover and a high shrub layer mainly composed of resprouting oaks. Pyrennean Oaks regenerate from resprouting, creating wood plots with dense undergrowth, where both the canopy and the shrub layer have a high foliage density (Costa et al. 1998). The presence of a well defined herbaceous cover indicated that the structure of such forest patches is well defined into three main height layers (i.e., ground, understorey and canopy), reflecting maturity. These mature territories had the features providing the highest caterpillar biomass in the preferred foraging substrates of tits (i.e., mature oaks and a shrub layer dominated by resprouting oaks; see Materials and Methods) and indicated a high temporal stability (i.e., three well defined height strata). Thus, highly yellow nestling plumage colours were found in favorable areas with respect to food availability, while this was not the case for bright plumages with relatively high reflectance at short wavelenghts. Ferns & Hinsley (2008) have also recently found that adult Great Tits living in low-quality habitats (small forest patches) exhibit yellow plumage with higher brightness.

According with expectations, different colour components of the yellow plumage of nestling Great Tits were related to different structural features of the breeding territories. Given that these colour components were calculated on the basis of a physiological model of avian perception, the effects found might be relevant in the context of intraspecific signaling. The rearing territories where nestlings had high plumage brightness were characterized by different features than those where nestlings had high relative long-wavelenght reflectance, but coincided with those where nestlings had high relative UV-blue plumage reflectance. The total brightness of yellow plumage (as a physical measurement not calculated from a visual model) is generated by the structure of feathers (Shawkey & Hill 2005, Shawkey et al. 2006), and reflectance at UV

wavelengths neither depends on the amount of carotenoids in this plumage type (Shawkey et al. 2006). Therefore, it seems likely that the structural components of nestling plumage colour were determined by the same habitat characteristics.

The relative reflectance at long-wavelengths in relation to other spectral regions (i.e. yellowness), but not brightness, reflects the carotenoid content of feathers (Saks et al. 2003, Shawkey et al. 2006). This is because, in unsaturated colours like that considered here, some absorbance of carotenoids occurs in spectral regions different from those of maximal absorbance, which makes long wavelength chroma the best indicator of pigment content (Andersson & Prager 2006). That short- (UV-blue) and long-wavelenght reflectance are associated with different habitat features, and features associated with higher relative short-wavelenght reflectance coincide with those associated with the brightest plumages, suggest that the UV-blue part of plumage spectra of nestling Great Tits mainly reflects feather structure. This is expected if it is considered that reflectance at UV-blue wavelengths are generated by the structural component of yellow plumage (Shawkey et al. 2006).

Experimental studies have demonstrated that the carotenoid-based colour of Great and Blue Tit nestlings is limited by the amount of carotenoids provided by the parents or in experimental supplementations (Fitze et al. 2003, Tschirren et al. 2003, Hadfield & Owens 2006) and by rearing conditions (Jacot & Kempenaers 2007). The expression of carotenoid-based colours depends on breeding territory quality because of differences in the availability of carotenoids or predation pressure (Hill 1988, Wolfenbarger 1999, Arriero & Fargallo 2006). Territory quality is also related to the expression of structural colouration (Keyser & Hill 2000), which indicates that generating this type of colours also depends on the nutritional status of birds (e.g. Keyser & Hill 1999, 2000, Siefferman & Hill 2005). However, pigmentary plumage colours also have structural components (Shawkey & Hill 2005). The present study shows for the first time that both structural and pigmentary components of the same colour patch of birds are related to different territory features. Since the expression of carotenoid-based plumage colouration may depend on the denaturation of certain proteins of the structural matrix of feathers (Blanco et al. 2005), it may be speculated that Great Tit nestlings reared in immature territories invest more in developing the structural elements of feathers and thus present a higher abundance of feather

proteins, which may impair the display of colour generated by feather carotenoids (Blanco et al. 2005).

On the other hand, we didn't know the sex of nestlings, which could represent a caveat for our results. The body mass and plumage brightness of Great Tits differ between sexes (Berthouly et al. 2008, Isaksson et al. 2008), which may affect the interpretation of our results concerning the body mass and plumage brightness of Great Tit nestlings if females would adjust clutch sex ratio in relation to territory characteristics, although these characteristics are also related to the nestling colour of the closely related Blue Tit, which is also sexually dichromatic, independently of sex (Arriero & Fargallo 2006). The same caveat should be considered for the relationship between Great Tit nestling colouration and cell-mediated immune response (see below), as sex differences in this response are found in some species (Dubiec et al. 2006).

In sum, the development of the different colour components seems to present a high phenotypic plasticity. This variability may respond to an adaptation to different environmental conditions like in other types of plumage colouration (Galván & Alonso-Alvarez 2009). As Great Tit nestlings showing nape yellow patches with high UV-blue reflectance get more food from their parents (Galván et al. 2008), it may be adaptive for nestlings reared in immature territories with low carotenoid availability to allocate more resources to develop the structural components of their feathers. Nestlings from mature territories, in contrast, may have plenty of carotenoids in their diets, which may allow them to develop yellower colourations that can have a signaling role after fledging (Tschirren et al. 2005). Badyaev et al. (2001) found that, in addition to colour, different components of pigmented plumage have different effects on the various components of individual fitness. The results of this study suggest a more complex communication system through interactions between feather pigments and structure. However, the UV-blue reflectance of the breast plumage, which is the patch considered here, does not seem to mediate parental favouritism in Great Tits like in the case of the nape patch (Galván et al. 2008), which may give support to the idea that the relationship between territory features and breast plumage colouration is mediated by a constraint rather than by an adaptive strategy. It is also possible that our results are due to a non-random genotype

distribution of the parents along the gradient in territory quality, and in this case the association between habitat features and colour could be due to genetic differences among broods rather than to phenotypic plasticity. Future experiments should test these ideas.

Relationships between plumage colour and nestling body mass and immune response

Nestling body mass, a measure of current physical condition (e.g. Keyser & Hill 2000), was related to territory features also denoting mature habitats, which supports the idea that the long-wavelenght component of yellow colour depends on the nutritional status of nestlings (Tschirren et al. 2003). However, and similarly to other studies (Hõrak et al. 2000, Pärn et al. 2005, Tschirren et al. 2005, Arriero & Fargallo 2006), nestling body mass was not related to any colour component, which confirms that habitat quality affects body mass and both structural (Keyser & Hill 2000) and carotenoid-based (Hõrak et al. 2000) plumage colour through different mechanisms (Hõrak et al. 2000).

Similarly, the cellular immune response of nestlings was not related to any colour component, but was related to structural vegetation features, which suggests that habitat quality may influence the immune response of nestlings and their plumage colour through different mechanisms. This seems contradictory because carotenoids may enhance the immune system of birds at the same time that it increases the expression of colour (Faivre et al. 2003, Alonso-Alvarez et al. 2004, Peters et al. 2004, Fitze et al. 2007). However, if territory quality affects the immune response and plumage colour of wild birds through different mechanisms (see above), the highest immune responses should not necessarily be found in mature (i.e., with high carotenoid availability) territories. In fact, Biard et al. (2006) have not found an increase in the T-cell mediated immune response of wild Great Tit nestlings after an experimental supplementation with carotenoids.

In any case, previous studies have shown that nestlings raised under poor environmental conditions are less immunocompetent (e.g. Hoi-Leitner et al. 2001, Arriero & Fargallo 2006), while our results are in the opposite direction. It is thus possible that in our case the most intense cellular immune responses were found in nestlings reared in territories with poor vegetation features because of the high levels of stress experienced by birds in such environmental conditions (Suorsa et al. 2003).

Conclusions

The findings of the present study show that structural and pigmentary components of a carotenoid-based plumage colour are related to different habitat features, although these results should be taken with caution since potential effects of sex and parent genotype, which were unknown in our case, cannot be discarded. While the structural components of plumage colour were positively correlated with rather immature habitats, the pigment-reflecting properties of feathers were associated with mature habitats with higher food availability, suggesting the existence of a high phenotypic plasticity in the development of different colour components. Associations of high nestling body mass with mature habitats support those findings, and the use of a visual perception model permits us to state that they might be relevant for the signaling system of Great Tits.

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STRESZCZENIE

**[Zależność między strukturą terytorium a kolo-
rem upierzenia, odpowiedzią immunologiczną
i masą ciała piskląt bogatki]**

Kolor upierzenia może zależeć od pigmentów
występujących w piórach jak i od struktury pióra,
która rozprasza światło. W badaniach anali-
zowano związek między strukturą terytorium
a dwoma komponentami koloru upierzenia,
odpowiedzią immunologiczna i masą ciała piskląt.
U 14–15 dniowych piskląt w 15 lęgach, przy pomo-
cy spektrofotometru mierzono odbijanie światła
w całym spektrum barw dla piór na piersi. Uzys-
kane wyniki grupowano ze względu na długości
fal biorąc pod uwagę ich zakres odbierany przez
oko ptaka, a następnie łączono je przy użyciu
analiz składowych głównych (Tab. 1). Analizowa-
ny kolor upierzenia bogatek rozdzielono na dwie
zmienne — barwę związaną z karotenoidami oraz
jaskrawość zależną od struktury pióra. W promie-
niu 25 m od gniazda szacowano pokrycie terenu
drzewami, krzewami oraz skałami, określano gru-
bość drzew, oraz udział poszczególnych gatun-
ków krzewów (młode dęby, janowiec *Genista flori-
da*, żarnowiec miotlasty). Na tej podstawie charak-
teryzowano środowiska ze względu na dostęp-
ność pożywienia. Odpowiedź immunologiczną
badano standardową metodą poprzez podanie
fitohemaglutyniny.

Barwa ani jaskrawość piór nie były związane
z masą ciała czy odpowiedzią immunologiczną

piskląt. Stwierdzono, że inne cechy środowiska wyjaśniają jaskrawość koloru, zaś inne barwę związaną z pigmentacją (Tab. 2). Barwa zależna od pigmentacji powiązana z karotenoidami odzwierciedlająca dostępność pożywienia była związana z dojrzałością drzewostanu, zaś jaskrawość była związana był z udziałem krzewów.

Masa ciała piskląt była większa w terenach ze starszymi dębami i licznymi żarnowcami (Tab. 3). Odpowiedź immunologiczna była pozytywnie skorelowana z udziałem janowca w środowisku, oraz negatywnie z wiekiem drzewostanu (Tab. 4).

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