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Habitat structure is associated with the expression of carotenoid-based coloration in nestling blue tits *Parus caeruleus*

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Abstract We investigated how the expression of carotenoid-based plumage coloration (lightness and chroma) in nestling blue tits *Parus caeruleus* is associated with forest structure in oak forests of central Spain. We found evidence of a reduced expression of carotenoid-based coloration in nestlings growing up in successional young and structurally simple forest territories. Our results suggest that breast feather coloration can be used as an indicator of nestling quality because nestlings with more intense yellow plumage coloration had larger body size and stronger immune responses to the injection of phytohemagglutinin (PHA). Given the association of forest structural complexity with carotenoid-based plumage coloration, our findings suggest that variation in habitat structure may have a significant impact on forest birds in their first stages of life which has implications for forest management practices.

Introduction

Forest structure, that is the complexity of vertical and horizontal organization in vegetation, is one of the most important attributes of forests from a management perspective, and may influence animal communities to a great extent (M. Hunter 1999; N.V.L. Brokaw and R.A. Lent 1999). Although primeval forests also show variation in structural complexity (H. John and B. Birks 2005), forest management may amplify structural variation in proximate

areas with similar natural characteristics such as exposition, altitude or successional status. Vegetation structure may hence influence the availability of refuges and nest sites for avian communities, distribution of food or microclimatic conditions (reviewed in N.V.L. Brokaw and R.A. Lent 1999), which might also affect the abundance and distribution of ectoparasites or arthropod vectors (S. Merino and J. Potti 1996).

Intensive forest management has been associated to a decrease in quantity and quality of resources for several forest avian species (L. Zanette et al. 2000), and to elevated levels of physiological stress as a consequence of poor nutritional status (P. Suorsa et al. 2003, 2004). The growing conditions experienced early in life can have long-lasting consequences on offspring quality and survival probabilities (J. Lindström 1999; P. Suorsa et al. 2003).

Signals based on carotenoid pigments are widespread in nature, and there are increasingly evidences of their use as honest indicators of individual quality in a great variety of species (M. Milinski and T.C.M. Bakker 1990; A.E. Houde 1997; G.E. Hill 1991). The expression of carotenoid-based characters has generally been interpreted in the context of sexual selection (M. Andersson 1994; G.E. Hill 1999). However, evidences of protection from predators by matching to the environment (A.H. Brush 1978; I. Slagsvold and J.T. Lifjeld 1985) or interspecific signalling of condition in parent–offspring communication (N. Saino et al. 2000) suggest that carotenoid-based signals can also arise by natural selection. Carotenoids are common pigments in nature, but they cannot be synthesized by animals (T.W. Goodwin 1984), and thus, must be obtained from the environment through the diet (V. Partali et al. 1987), for which environmental factors may have a large influence on carotenoid-based signals (G.F. Grether et al. 1999). Animals living in carotenoid-poor environments have less intense coloration in their secondary sexual characters (e.g., J.A. Endler 1980; I. Slagsvold and J.T. Lifjeld 1985; P. Hõrak et al. 2001), and the addition of these pigments in the diet, may enhance the coloration of the ornament (e.g., A. Kodric-Brown 1989; G.E. Hill 2002; B. Tschirren et al. 2003).

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Besides the function in intraspecific and interspecific signalling, carotenoid pigments can be used for a variety of physiological processes such as immune defense or detoxification activity (G.A. Lozano 1994; V.A. Olson and I.P.F. Owens 1998; T. von Schantz et al. 1999). Proliferation of T- and B-lymphocytes, stimulation of T-cell function and antibody production among others, have been attributed to different kinds of carotenoid pigments (reviewed in A.P. Møller et al. 2000). An increasing number of studies show an association between variation in immune activity and the expression of carotenoid-based ornaments, reinforcing the function of these ornaments as reliable indicators of individual condition (e.g., J.D. Blount et al. 2003; K.J. McGraw and D.R. Ardia 2003; L. Saks et al. 2003).

The vast majority of studies about the expression of carotenoid-based ornaments have focused on adult individuals, and less attention has been paid to the meaning and implications of the expression of these traits in juveniles (N. Saino et al. 2000; B. Tschirren et al. 2003). In several bird species, young individuals also show conspicuous ornaments (e.g., B.E. Lyon et al. 1994; P. Hørak et al. 2001) that could be seen as correlated traits indirectly evolved through selection on adult ornaments (M. Andersson 1994). However, the absence of correlation between nestling and first year carotenoid-based plumage reported in the great tit *Parus major* (P.S. Fitze et al. 2003a) suggests that nestling plumage coloration may have evolved under different selection pressures than adult coloration (P. Hørak et al. 2001). The expression of costly ornaments in nestlings may have evolved as a result of parent–offspring conflict on resource distribution (E.A. Krebs and D. Putland 2004). Parental food allocation based on the expression of ornamental signals in nestling has been reported in American coots (B.E. Lyon et al. 1994), suggesting a considerable role of this character in parent–offspring communication.

Blue and great tits *Parus caeruleus*, *P. major* are among the few bird species where colorful plumage based on carotenoid pigments is expressed during the nestling period (e.g., A. Johnsen et al. 2003; P.S. Fitze et al. 2003b). The yellow feather coloration in these *Paridae* tits is due to lutein and zeaxanthin ingested with the food, mainly caterpillars (V. Partali et al. 1987), and is known to be condition-dependent (P.S. Fitze et al. 2003a). Thus, nestling plumage color in these species might be signaling phenotypic quality to parents to attract a higher investment during the period of postfledging dependence.

In the present study, we use the well-known blue tit *P. caeruleus* to examine whether the expression of carotenoid-based coloration in nestlings differs as a function of habitat structure at the spatial scale of breeding territory. If the presence or abundance of invertebrates (i.e., main source of carotenes for tits) is associated to forest characteristics (L. Zanette et al. 2000), we predict nestling coloration to be associated to habitat complexity indexes. Similarly, if forest structural complexity promotes habitat-dependent variation in the allocation to carotenoids to physiological functions, we expect to find association

between the expression of plumage coloration and immune function. We explore possible causes of individual variation in color expression in nestlings, and we test whether nestling plumage coloration may function as indicator of individual quality.

Materials and methods

The study was conducted during the breeding season of 2002 in deciduous forests of Pyrenean oak, *Quercus pyrenaica* Willd., in central Spain (40°49'N, 3°56'E). Pyrenean oak forests are the commonest deciduous woodlands of montane areas in central Spain (M. Costa et al. 1998), and the preferred breeding habitat for the blue tit in this region (M. Diaz 2003). The blue tit is a small (11 g) hole-nesting passerine of European woodlands which adapts readily to breed in nest boxes (S. Cramp and C.M. Perrins 1993). Egg laying in central Spain typically begins in early April, the mean clutch size is nine eggs, and the number of fledglings averages seven (J. Potti et al. 1988; J. Moreno et al. 1996). A total of five hundred nest boxes were installed in five different 20 ha-forest patches in a grid with 30–50 m between adjacent boxes. The forest patches were located between 1,100 and 1,500 m a.s.l. with an average interarea distance of 18±3.3 km.

Nest-boxes were checked for occupation by blue tits, and the dates of clutch initiation, clutch sizes, and number of fledged young were determined. On day 15, after hatching (hatching date = 0), nestling tarsus length was measured to the nearest 0.01 mm with a digital calliper, and their body mass was recorded with an electronic balance (Tanita #1479, UK Ltd.) to the nearest 0.1 g. Breeding birds were captured with nest box traps on day 7–8 after hatching and similarly measured.

Habitat structure

Pyrenean oak forests in this region of central Spain are, to a significant extent, solely comprised of Pyrenean oak *Quercus pyrenaica* in the tree layer. The structural features of the forest reveal to a great degree the successional stage of the forest, with young successional stages represented by simple structural attributes (N.V.L. Brokaw and R.A. Lent 1999). For the purpose of the study, structural characteristics were estimated within a radius of 25 m around the nest-box. Because the study aimed to evaluate the relationship between forest structural characteristics and the expression of carotenoid signals in nestlings, the scale of breeding territory was considered appropriate to examine variation in plumage coloration (D.W. Morris 1987; J.F. Urban 2005). This area was considered representative of the variability in habitat structure of the home range of breeding pairs (E. Svensson and J.-Å. Nilsson 1995), given the uniformity of managed oak forest in the region. However, the spatial scale of forest plot is also considered and controlled in the analysis (see “Statistical analyses” section).

The structural variables chosen included estimates of vertical profiles (shrub and tree layer height) as well as horizontal profiles (density of juveniles <10 cm dbh, density of old oak trees >30 cm dbh, and the average diameter of the five thickest trunks at breast height). These variables were the ones that best revealed the variation in habitat structure at the scale selected in the study (G.H. Orians and J.F. Wittenberger 1991). A principal components analysis combining these variables was used to calculate a single habitat structure index. The first principal component (PC1) explained 50.5% of the variation (eigenvalue=2.53), and defined a gradient of “forest structural complexity”. Lower values of the factor represented successional young and heavily managed forest plots (reduced density of thick trunks, lower average tree height, and thinner trunk diameter). Factor loadings: shrub height = 0.293, tree height = 0.784, trunks 5–10 cm = -0.08, trunks >30 cm = 0.812, average trunk diameter = 0.898. Forest structural complexity (PC1) differed significantly among the five forest patches ($F_{4,256}=108.32$, $p<0.001$; Alameda -0.94 ± 0.06 , Navacerrada -0.53 ± 0.07 , Miraflores 1.09 ± 0.08 , Navafria 0.32 ± 0.10 , Pinilla -0.70 ± 0.06).

Plumage color quantification

Plumage color was measured using a portable spectrophotometer (MINOLTA CM2600d; wavelength range 360–740 nm), and characterized according to the LCH color space defined by the International Commission of Illumination (Commission Internationale de l’Eclairage, CIE). L^*h^*c describe color characteristics and has been used in numerous studies characterizing avian plumage (e.g., J. Figuerola et al. 1999; J.C. Senar et al. 2002), which allows comparisons with previous studies. The coordinate “L” specifies the lightness (also called brightness) of a color and varies from 0 (black) to 100 (white). “h” specifies hue, which refers to the saturation or purity of the dominant frequency of the light. The coordinate “c” specifies chroma (also called color saturation), which corresponds to color purity and monochromatism. For the purpose of this study, only chroma and lightness were analyzed (J.A. Endler 1990).

Nestling plumage color was measured when they were 15-day old. Feather color was measured with an aperture of 3 mm diameter, placing directly the spectrophotometer on top of the feathers on one side of the sternum of the nestling. The repeatability of the color measurements using the portable spectrophotometer directly on breast feathers was calculated taking three measurements of color on the same area on one side of the sternum. The intraindividual repeatability of color measurements was very high (lightness, $r=0.95$, $p<0.001$; chroma, $r=0.93$, $p<0.001$).

Molecular sexing

Molecular sexing of nestlings was conducted by using primers P2 and P8, according to the method by R. Griffiths et al. (1998). Two homologous genes are amplified with this method: CHD1Z occurring in both sexes, and CHD1W occurring only in the W chromosome carried by females. The PCR products were separated on 1.5% agarose gel containing ethidium bromide, and visualized under UV light. A total of 367 nestlings from 41 broods were sexed with this method. Samples taken from six breeding adults were correctly sexed using this method. Nine nestlings sexed from blood samples were recaptured the next breeding season as adults. In all cases, the sex determination using genetic markers agreed with that determined in the field.

Assesment of immune function

Cell-mediated immune response of nestlings was measured with the phytohemagglutinin injection assay (see J.E. Smits et al. 1999, for details of the method). Fourteen-day old nestlings were injected with 0.2 mg of phytohemagglutinin (PHA) in 0.04 ml of saline solution in the left patagium after measuring its thickness at the point of injection. After 24 h, a new measurement of the thickness of the patagium at the same point was taken. T-cell immune response was estimated as the difference between initial and final measurements. The repeatability of patagium measurements was high and significant, both for initial ($r=0.89$, $p<0.001$) and final ($r=0.86$, $p<0.001$) measurements.

Statistical analyses

Individual variation in the expression of carotenoid-based coloration was analyzed using General Linear Models in SAS statistical software (R.C. Littell et al. 1996), including chroma and lightness as response variables in two separate models. The models used Randomized Complete Block Design (R.C. Littell et al. 1996), where site and nest id were considered as blocks (and random effects), and nest id was nested within site. As explanatory variables we included nestling sex, brood size, forest structural complexity (PC1), hatching date, plumage color characteristics of male and female parents, and nestling morphological traits. To account for spatial autocorrelation—i.e., nest within a forest patch not independent from each other—(H.H. Wagner and M.J. Fortin 2005) we adjusted models for within-site spatial covariance by estimating the nugget, partial sill and range parameters of the semivariogram explaining spatial correlation within sites (R.C. Littell et al. 1996). Nest coordinates (i.e., latitude and longitude) were used. Four spatial models

for explaining covariance structure were compared (Gaussian, exponential, power, and spherical; R.C. Littell et al. 1996) to an independent errors model that did not adjust for spatial covariance. The model which better explained the structure of our data was the one not including spatial corrections, and is the one presented in the results. Some of the explanatory variables could covariate, thus we fitted their effects to the observed data following forward and backward stepwise procedures, testing the significance of each variable one by one, and adding (forward) or removing (backward) only the variable that resulted in the largest increase in model fit. The result is the most adequate model for explaining the variability in the response variable coinciding in both forward and backward processes. Residuals from all models were checked for normality (Kolmogorov–Smirnov, all $p > 0.05$). The equality of variances of dependent variables was tested when using fixed factors in the model (all $p > 0.16$). Degrees of freedom were calculated by the Satterthwaite approximation (R.J. Freund et al. 1986).

Not all parents were caught, therefore sample sizes differed in the analyses. The skin test for immune assessment was only performed in 33 complete broods, thus sample sizes were reduced in this analysis. Means \pm SE are given throughout.

Results

Male and female nestlings differed in their morphological measurements 15 days posthatching. Male nestlings were heavier and had longer tarsi than their female nest-mates (Table 1). Sexual differences were also found in the expression of carotenoid-based breast plumage coloration. Males were more chromatic than females and showed a plumage with higher lightness (Table 1).

Plumage chroma was positively correlated with lightness ($\beta = 0.75$, $p < 0.001$, $n = 367$). Yellow chroma and lightness were positively correlated with forest structural complexity-PC1 (Table 2). Both chroma and lightness were higher in nestlings growing up in breeding territories with higher density of old trees and greater average of tree height and trunk diameter (Table 2, Fig. 1). Nestling plumage color characteristics were also positively associated to hatching date (Table 2). Nestlings hatched late in the breeding season had more intense plumage coloration

Table 1 Sex differences (mean \pm SE) in morphological measurements and plumage color characteristics

Variable	Males	Females	$F_{1, 318}$	p
Body mass (g)	10.92 \pm 0.05	10.55 \pm 0.05	66.50	<0.001
Tarsus length (mm)	16.74 \pm 0.04	16.30 \pm 0.04	135.69	<0.001
Yellow chroma	29.67 \pm 0.33	27.48 \pm 0.36	41.98	<0.001
Lightness	68.77 \pm 0.39	67.25 \pm 0.37	15.37	<0.001

Mixed model ANOVA with nest as random factor ($n = 367$ nestlings; 195 males and 172 females)

Table 2 Variables related to nestling breast plumage coloration

Variable	df	Estimate	F	p -value
Chroma				
Forest structural complexity	1,42.7	1.77	14.66	<0.001
Hatching date	1,43.1	0.20	9.84	0.003
Brood size	1,45.5	-0.38	3.66	0.062
Sex	1,360	-1.89	16.98	<0.001
Tarsus length	1,337	1.67	13.76	<0.001
Lightness				
Forest structural complexity	1,43.4	1.66	9.02	0.004
Hatching date	1,44.4	0.18	5.63	0.022
Sex	1,360	-1.32	6.27	0.013
Tarsus length	1,348	1.15	4.82	0.029

Mixed model ANCOVA with nest within site as random factor. Type 3 of fixed effects with df error computed using Satterthwaite method. Variables not significantly contributing to explain individual variation in colour were removed from the final models (chroma: all non-significant p -values > 0.14 ; lightness: all non-significant p -values > 0.2). No significant interactions were found among the explanatory variables

independently of habitat characteristics. Larger broods tended to have less chromatic coloration (Table 2), and nestling morphological measurements showed that nestlings with longer tarsi had more intense breast plumage color (Table 2). Nestling body mass and color characteristics of parents were not significantly associated to the expression of carotenoid-based color in nestlings and then excluded from the final models (all $p > 0.14$). Residuals of the model showed a normal distribution ($W = 0.99$, $p = 0.8$).

Plumage characteristics were also related to the ability to mount an immune response. Individual data showed a positive association between plumage lightness and immune response ($F_{1,49,6} = 5.25$, $p = 0.026$, Fig. 2). Cell-mediated immune response was not related to forest characteristics ($F_{1,31} = 0.09$, $p = 0.771$), nestling body mass ($F_{1,42,5} = 0.37$, $p = 0.543$), or sex ($F_{1,76} = 2.31$, $p = 0.132$), and no significant interactions were found in the analysis.

Discussion

We found evidence that habitat characteristics are associated with the expression of carotenoid-based plumage coloration during the nestling period. Habitat effects on the expression of carotenoid-based coloration in nestling tits were reported by I. Slagsvold and J.T. Lifjeld (1985) in a study of Great tits growing up in deciduous and coniferous forests of Norway. Their study is one of the most relevant contributions showing an association between habitat characteristics (i.e., amount of deciduous foliage around the nest) and plumage coloration in nestling tits (I. Slagsvold and J.T. Lifjeld 1985; but see also Hōrak et al. 2000). Our results show that Blue tit nestlings growing up in young and degraded forest habitats exhibit less saturated yellow breast plumage. Ample clear-cuts and removal of old trees as a result of management practices may have resulted in small-scale variation in the availability of

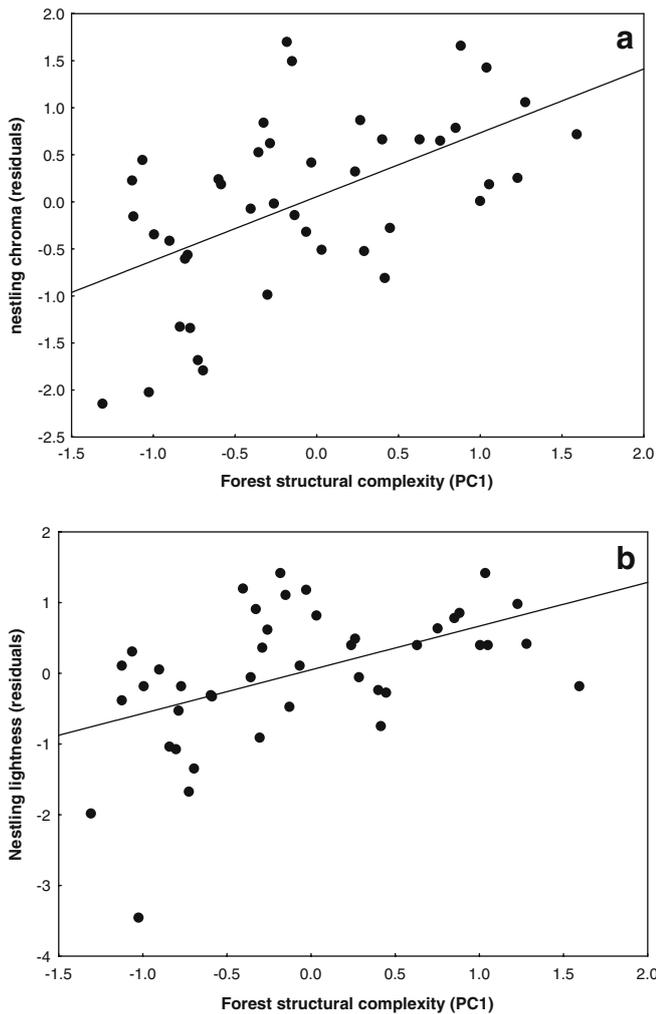


Fig. 1 Relationship between forest structural complexity and characteristics of nestling plumage coloration (**a** yellow chroma, $y=0.06+0.68x$; **b** lightness, $y=0.05+0.62x$). The graphs represent the residuals of the regression models for chroma and lightness including the variables which significantly contributed to explain the variance observed (for chroma: brood size, hatching date, brood sex-ratio, and mean tarsus length; for lightness: hatching date, brood sex-ratio, and mean tarsus length). Mean values per brood are represented

carotenoid-rich food for the blue tit within the same type of deciduous forest.

Geographical or temporal variation in plumage coloration has been previously reported in different avian species, mostly associated to food availability (e.g., I. Slagsvold and J.T. Lifjeld 1985; G.E. Hill 1993; P. Hørak et al. 2000; J.C. Senar et al. 2003). However, food availability seemed not to be a limiting factor during the nestling period in our population because nestling body size before fledge was not associated to forest structural characteristics (E. Arriero et al. 2006). Yet, the negative trend between brood size and plumage coloration found in our study suggests that carotenoid availability could be limiting in large broods (P. Hørak et al. 2000; B. Tschirren et al. 2003), but might not be the main factor explaining individual variation in plumage characteristics in the offspring (P.S. Fitze et al.

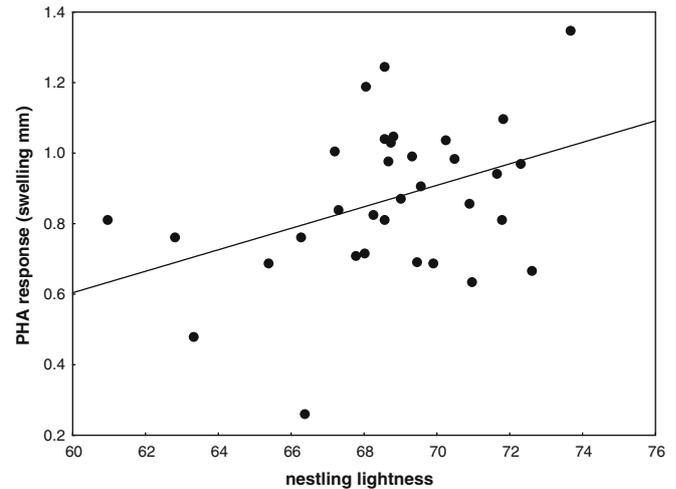


Fig. 2 Cell-mediated immune response (PHA skin test) in relation to plumage lightness. Mean values per brood are represented ($y=-1.22+0.03x$)

2003a). Besides, brood size was not associated to forest structural complexity in our study population (E. Arriero et al. 2006).

Food limitation, in relation to vegetation structure in the deciduous forests of our study site, seems to have a greater impact on initial phases of reproduction (E. Arriero et al. 2006). A seasonal increase in the availability of carotenoid-rich food for laying females as the abundance of lepidopteran increases (J.J. Sanz 2001) could also lead to seasonal variation in maternal allocation of carotenoids to the egg yolk (J.D. Blount et al. 2000; N. Saino et al. 2003), and thus explain the positive association between nestling color and timing of breeding found in our study. An active increase in the allocation of carotenoids might also be promoted as a mean to up-regulate and stimulate the immune function of late nestlings exposed to higher risk of parasite infections (S. Merino et al. 2000). Nevertheless, forest structural characteristics may explain other aspects of nestling developmental environment, and thus promote habitat-dependent allocation of carotenoid pigments to different physiological functions (V.A. Olson and I.P.F. Owens 1998; T. Eeva et al. 1998; A.P. Møller et al. 2000). Microclimatic conditions associated to forest structure (M. Hunter 1999) might influence for example nestling metabolic expenditures, or the thermal quality of a particular breeding territory for ectoparasites or arthropod vectors (R. B. Huey 1991). Thus, differences in the risk of parasite infections or in the physiological state of nestlings associated to degradation of forest habitats (P. Suorsa et al. 2003, 2004) could influence the expression of this condition-dependent trait (reviewed in A.P. Møller et al. 2000).

The function of color signalling in nestlings still remains contentious (A. Johnsen et al. 2003; B. Tschirren et al. 2005). Our results show that nestlings with longer tarsus had more intense plumage coloration, suggesting that carotenoid-based coloration can be a reliable indicator of individual quality through its association with a fitness-

related trait (reviewed in S. Gebhardt-Henrich and H. Richner 1998). Plumage color might be acting in parent-offspring signalling, either at the nestling stage or after fledging (but see B. Tschirren et al. 2005). A differential parental feeding effort based on color signalling could be possible (B.E. Lyon et al. 1994; F. Götmark and M. Ahlström et al. 1997; N. Saino et al. 2000) also in tits, which remain with their parents for about 3 weeks after fledging. In addition, plumage lightness was positively associated to nestling immune function. A higher T-cell-mediated immune response, as measured in vivo by the response to the inoculation of phytohemagglutinin (PHA), has been shown to be a good predictor of offspring survival (e.g., P. Christie et al. 1998; J. Moreno et al. 2005; M. Cichoń et al. 2005; but see review by A.P. Møller and N. Saino 2004). In contrast, nestling body mass was not significantly associated to the expression of plumage coloration (but see A. Johnsen et al. 2003; B. Tschirren et al. 2003). Body mass might thus be a correlate of other traits really associated with the expression of carotenoid-based coloration in our study population (see also I. Slagsvold and J.T. Lifjeld 1985).

On the other hand, despite the parent-offspring signaling role of nestling plumage coloration, the sexual differences found in plumage color in 15-day-old nestlings could be also explained in the context of sexual selection. Both color components, lightness and chroma, were higher in male nestlings than in females. Sexual differences could be explained as favoring early mate attraction or male-male competition for territories during early autumn (A. Johnsen et al. 2003), but it could also be the consequence of sexual variation in the physiological processes related to the utilization of carotenoids (K.J. McGraw et al. 2003).

The absence of correlation between parent-offspring color characteristics supports the idea that the two plumages might be different traits, and environmental conditions have a strong effect on nestling plumage (P.S. Fitze et al. 2003a), besides the possible heritable variation in physiological processes involved in pigment absorption, transportation or deposition in feathers (K.J. McGraw et al. 2003). In addition, the fact that adult male coloration was not associated to structural characteristics of the breeding territories (data not shown), and that there were not significant relationship between male and nestling plumage color, reduce the possibility that the results were obtained in a scenario of different blue tit phenotypes occupying territories of different quality (see R.V. Alatalo and A. Lundberg 1986). Nestling plumage characteristics seem more appropriate to evaluate association with habitat features at the scale of breeding territory because we have no information about the characteristics of the areas where adults moulted their plumage.

To conclude, the expression of carotenoid-based signals early in life has shown to indicate nestling quality and survival probabilities through its association with the immune function. Given the association between forest structural characteristics and the expression of carotenoid-

based plumage coloration, plumage coloration may be used as an indicator of habitat quality. Our results suggest that variation in habitat structure may have an important impact on offspring condition for forest birds, which has implications for forest management practices.

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