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Sexual, seasonal, and environmental variation in plasma carotenoids in great tits, *Parus major*

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In many birds, carotenoids have dual functions as irreversible plumage pigments and as physiologically essential vitamins and antioxidants. They must be obtained through the diet and may therefore be a limiting resource, a constraint that is likely to vary with factors such as sex, habitat, and time of year. In the present study, we investigated signs of carotenoid limitation in great tits, *Parus major*, in relation to sex, season, year, and within an urban versus a rural habitat. The two main carotenoids, lutein and zeaxanthin, were analysed by high-performance liquid chromatography in the plasma and in the yellow carotenoid-based breast feathers. We found that plasma carotenoid concentrations were significantly influenced by sex, season, and year, but not by urban versus rural habitat. At moult, plasma concentration was positively correlated with feather pigmentation, independent of body condition and sex. During the breeding season, however, circulating carotenoid concentrations were negatively related to the feather pigmentation (i.e. from previous autumn moult). We suggest that great tits are carotenoid deprived before leaf emergence, and that carotenoid utilization and limitations are sex-specific, but that there are neither any obvious honesty-maintaining costs of pigmentation, nor any fitness consequences of the colour variation.

ADDITIONAL KEYWORDS: allocation conflict – antioxidants – caterpillars – feather pigmentation – lutein – plumage coloration – pollution – zeaxanthin.

INTRODUCTION

Carotenoid-based colour variation in birds has received considerable attention in evolutionary ecology and sexual selection (Andersson, 1994; Bennett & Owens, 2002; Hill & McGraw, 2006). A major reason for this is the potential trade-off between health and (irreversible) plumage pigmentation (Lozano, 1994; Olson & Owens, 1998; von Schantz *et al*., 1999), arising from the internal functions of carotenoids as antioxidants, protectors of cell membranes, DNA and lipids from oxidative damage (Sujak *et al*., 1999; Bianchini *et al*., 2000). Consequently, as the predictions go, the more carotenoids needed for such functions, the less carotenoids available for ornamentation (Olson & Owens, 1998).

The possible trade-off ultimately arises from the fact that birds and other animals can not synthesize carotenoids *de novo* ('from scratch') and are therefore dependent on the carotenoid content in their diet (Goodwin, 1984; Partali *et al*., 1987). Like other nutritional constraints, carotenoid availability may vary with spatial and seasonal habitat changes and associated variation in food quality and quantity (Slagsvold & Lifjeld, 1985; Hill, 1992; Hill, 1995a, b; Eeva, Lehikoinen & Sunell, 1997; Bortolotti *et al*., 2000; Negro *et al*., 2001). Carotenoid intake is, however, not the only determinant of which and how much carotenoids eventually become available for, for example, plumage pigmentation (but see Hill, Inouye & Montgomerie, 2002). Several physiological factors can also be additional or even main constraints, namely through variation in uptake efficiency in the gut or amount of lipoproteins for transportation (Brush, 1990; McGraw & Hill, 2001; Tella *et al*., 2004; McGraw, 2006).

The main carotenoids utilized by birds are xanthophylls (oxygenated carotenoids), such as lutein and zeaxanthin, which are ubiquitous in yellow bird

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plumage (Stradi, 1998; McGraw, 2006). To a lesser extent, birds also assimilate carotenes (pure hydrocarbons), such as β-carotene. Despite the substantial literature on sexual and natural selection on carotenoid-based coloration, however, there is surprisingly little empirical data available with respect to the central ecological and physiological factors influencing the acquisition and utilization of carotenoids (McGraw, 2006). In the present study of the great tit, *Parus major*, a small palearctic passerine with yellow, carotenoid-pigmented ventral plumage, we use high-performance liquid chromatography (HPLC) analysis of blood plasma to investigate sexual and environmental (i.e. season, year and urban versus rural habitat) variation in circulating carotenoid levels. We also investigate if and how plasma levels during moult (September) reflect incorporation of carotenoids into the ventral plumage, and its relationship with condition and sex.

From September (i.e. during moult) to April, great tits are omnivores, eating various insects, spiders, and seeds (Cramp & Perrins, 1993), most of which generally are poor in carotenoids (Latscha, 1990). After leaf emergence and during breeding (May to June), however, the main food sources are different *Lepidoptera* caterpillars, which are rich in carotenoids (Partali *et al*., 1987). Thus, we predict that seasonal constraints on carotenoid availability could result in carotenoid limitation (e.g. during the September moult and before leaf emergence).

Previously, we have shown that great tits living in urban habitats have an increase in antioxidant usage, as indicated by the glutathione antioxidant system (Isaksson *et al*., 2005), a pattern that was accompanied by paler carotenoid-based plumages in urban areas (Eeva, Lehikoinen & Ronka, 1998; Hõrak *et al*., 2000; Isaksson *et al*., 2005). Thus, the carotenoid limitation may be most pronounced in the urban habitat, which may be revealed by lower plasma levels of carotenoids in urban birds. On the other hand, if carotenoid access is not severely limited, urban and supposedly more stressed birds may respond to the environmental stress by increased intake, uptake or transport of carotenoids, which thus (at least when carotenoid availability allows) may result in higher plasma concentration in urban birds.

Moreover, if we assume that carotenoids are limiting during all seasons, we expect sexual differences in plasma carotenoids to be most pronounced at breeding or more precisely after egg laying, during which the females has allocated large amounts to the egg yolks, where carotenoids provide protection for the embryo, but also to the hatchling (Blount, Houston & Møller, 2000; Surai, 2002; Karadas *et al*., 2005). Thus, during breeding, this may be reflected in lower concentrations in female plasma compared to males.

MATERIAL AND METHODS

Study species and field site

The great tit, *Parus major*, is a common passerine throughout Europe and Asia. Both males and females have yellow carotenoid-based ventral plumage, mainly from the dietary xanthophylls, lutein and zeaxanthin (Partali *et al*., 1987; Stradi, 1998). Together with β-carotene, these appear to be the main carotenoids in great tit diet (Partali *et al*., 1987).

The urban and rural great tit populations investigated in the present study breed in nest boxes in similar deciduous forests in the south-west of Sweden. Areas designated as urban (Änggården, Slottsskogen, and Gunnebo) (i.e. with high exposure to air pollution; Kindbom *et al*., 2001) are located within Göteborg city limits. The rural areas (Högås, Hamra, Gräppås, and Råön) are located in the countryside, 40–50 km south of Göteborg along the coast, where a nearby permanent air analysis station has documented substantially lower levels of especially nitric oxides, sulphur dioxide, and soot, compared to the urban measurements (Kindbom *et al*., 2001).

Birds were trapped in the nest boxes during 'prebreeding' (March to April) and 'breeding' (May to June), whereas 'postbreeding' birds (September to October) were caught in mist nets. The birds were sexed according to plumage characteristics (Svensson, 1992), weighed (±0.1 g) with a Pesola spring balance and tarsus length was measured (±0.1 mm) with a sliding caliper. A heparinized syringe was used to draw 150–200 μL blood from the neck vein. The blood was kept on ice in the field (1–4 h) until further handling in the laboratory. Samples and measurements were collected over 3 years: 2002, 2003, and 2004 (for sample sizes, see Table 1).

Extraction of carotenoids from plasma and feathers

The blood samples were centrifuged at 260 *g* for 10 min, whereafter the separated plasma (60–130 μL) was stored at –80 °C until analysed. The day before the analysis, 20 μL of plasma was mixed with 380 μL of acetone and then frozen overnight at –80 °C. In total, plasma samples from 241 individuals were analysed, distributed as shown in Table 1.

The samples were centrifuged at 14 000 *g* for approximately 5 s. The liquid phase was filtered through a 0.2 μm syringe filter (GHP Acrodisc 13 mm; Pall Gelman Sciences Inc.), and subsequently evaporated in a vacuum centrifuge (Savant DNA 120). The samples were not allowed to dry completely to minimize the exposure to air. Following resuspension in 100 μL of the mobile phase (70 : 30 acetonitrile : methanol, v/v), samples were immediately analysed by HPLC.

Table 1. Number of plasma samples analysed by high-performance liquid chromatography and their distribution among different habitats (urban/rural), seasons (pre/during/post breeding) and years

	Urban						Rural					
	Pre		During		Post		Pre		During		Post	
Year	F	M	F	M	F	M	F	M	F	M	F	M
2002	–	–	18 (8)	24 (9)	–	3	–	–	–	10 (4)	–	–
2003	5	5	16	17	–	–	14	11	17	26	–	–
2004	–	1	–	–	4 (4)	5 (4)	–	19	31	7	5 (5)	3 (3)

The numbers in parentheses are the feather samples analysed.
M, males; F, females.

Following a protocol modified from Stradi *et al.* (1995), feathers were washed with hexane and weighed to the nearest 0.1 mg (Mettler Toledo AB54-S). Thereafter, approximately 1 mg of coloured barbs were trimmed off with surgical scissors and homogenized in 3 mL of methanol in a Retsch MM2000 micronizer with ZrO containers, at 27 Hz for 15 min. The white keratin residue was filtered off with a 0.2 μm syringe filter (GHP Acrodisc 13 mm). After evaporating the methanol, the residue was resuspended in 150–200 μL of acetone, placed at –80 °C overnight, and filtered (as described above) for a second time, followed by evaporation of the acetone. The carotenoid residue was finally dissolved in 100 μL of the mobile phase (70 : 30 acetonitrile : methanol, v/v) and immediately analysed using HPLC.

High performance liquid chromatography (HPLC)

Depending on the sample colour (an approximate estimate of carotenoid concentration), 20–40 μL of the sample was injected with isocratic mobile phase (see above), through a 100 μL loop into a RP-18 column (ODS-AL, 50 × 4.0 mm i.d., YMC Europe GmbH), fitted on a ThermoFinnigan HPLC system with a PS4000 ternary pump, AS3000 auto sampler, and UV6000 diode-array UV/VIS detector. The column temperature was maintained at 30 °C with a flow rate of 0.6 mL min^{-1}. The run time was set to 15 min. Two-dimensional (at 450 nm) and three-dimensional (300–600 nm) chromatograms were obtained and analysed with ChromQuest 4.0 software (ThermoFinnigan). Major pigment fractions were identified and quantified by comparison with internal standards and calibration curves, respectively, of lutein (β,ε-carotene-3,3′-diol) and zeaxanthin (β,β-carotene-3,3′-diol), provided by Roche Vitamines Inc. All other chemicals (methanol, *n*-hexane, acetonitrile, and acetone) were obtained from VWR International.

Statistical analysis

Data and residuals were checked for normal distribution and the best Box Cox transformation ([(carotenoid concentration$^{0.2}$) – 1]/0.01765) was used for the plasma carotenoid concentration. A general linear model with backward elimination ($P > 0.25$; Quinn & Keough, 2002) was run with plasma carotenoid concentration as the dependent variable with independent variables sex, season (pre-, during, and postbreeding), year, habitat (urban and rural), and condition [calculated as ln mass/(3 × ln tars)]. To minimize the model, we decided not to include age. In the second model, we used feather carotenoid concentration as the dependent variable with independent factors sex, habitat (urban/rural), and condition. First-order interaction terms were initially included but removed if clearly nonsignificant ($P > 0.25$). All data are means ± SE. All analyses were performed in JMP, version 5.1 (SAS Institute Inc.).

RESULTS

Circulating carotenoids

Plasma carotenoid concentration was highly variable among samples, with significant effects of sex, season, and year, but not of body condition or urban versus rural habitat (Table 2). In early spring, before leaf emergence, great tits exhibited lower levels of circulating plasma carotenoids than later during chick feeding and during the subsequent early fall (least square mean, LSM; prebreeding: 18.18 μg mL^{-1}, during breeding: 38.53 μg mL^{-1}, postbreeding: 45.78 μg mL^{-1}; Table 2). Females had significantly lower overall levels than males (LSM; female: 7.65 μg mL^{-1}, male: 28.72 μg mL^{-1}). As evident by the significant interaction terms (Table 2), the sexes differed in the pattern of seasonal change; whereas females started with the lowest prebreeding levels and increased steeply to a postbreeding maximum,

males varied less over the year with peak concentrations during breeding (Fig. 1). Males and females showed the same overall yearly differences, with significantly higher plasma concentrations in 2003 than in the two other years.

Urban birds were in poorer condition (i.e. relative body mass) than rural birds (habitat; $F_{1,229} = 9.59$, $P = 0.002$), and males were in better condition than females (sex; $F_{1,229} = 4.77$, $P = 0.030$). Individual variation in body condition was, however, not related to plasma carotenoid concentration (Table 2) and the significantly higher body condition in 2004 ($F_{2,229} = 23.56$, $P < 0.0001$) did not coincide with the year of exceptionally high plasma carotenoid levels (2003).

Plumage carotenoids

Feather carotenoid concentration was unrelated to sex (mean total carotenoid concentration (μg) per gram breast feather; female: 33.61 ± 4.73 μg g^{-1} feather, $N = 9$; male: 33.95 ± 3.97 μg g^{-1} feather, $N = 7$) and condition, and only a nonsignificant tendency of an environmental (urban/rural) effect was retained in the model (habitat; $F_{1,14} = 2.92$, $P = 0.109$, $N = 16$). Because of unbalanced data among years with regard to environment (Table 1), only data from 2004 were included in the analysis. In a model pooling the habitats and years 2002 and 2004, and using feather carotenoid concentration as the response variable, we found that feather pigmentation was significantly and positively related to plasma carotenoid concentration ($F_{1,31} = 5.01$, $P = 0.033$) as well as to its interaction with season ($F_{1,31} = 10.49$, $P = 0.003$). Finally, a correlation between deposited and circulating carotenoids was also indicated by a bivariate relationship between feather carotenoid concentration and plasma concentration during moult (i.e. 'postbreeding') ($F_{1,14} = 8.42$, $P = 0.012$, $N = 16$). By contrast, during breeding the corresponding relationship was weakly negative ($F_{1,17} = 4.22$, $P = 0.056$, $N = 19$).

Table 2. Results from a general linear model with plasma carotenoid concentration (μg mL^{-1}) as the dependent variable

Source	d.f.	*F*-ratio	*P*
Sex	1	7.78	0.006
Season	2	27.18	< 0.0001
Year	2	37.59	< 0.0001
Condition	1	1.84	0.176
Sex × season	2	8.20	< 0.001
Sex × year	2	8.63	< 0.001
Condition × year	2	4.19	0.016

Season includes pre-, during, and postbreeding. Environment (urban/rural) and interactions not shown in the table were excluded from the model. Error degres of freedom (d.f.) = 217.

DISCUSSION

During early spring, before leaf emergence, great tits had significantly lower concentrations of plasma carotenoids than later, at the time for chick feeding, which coincides with the appearance of foliovorous, carotenoid-accumulating *Lepidopteran* caterpillars (Partali *et al*., 1987). This indicates that, during winter and spring (before leaf emergence), carotenoids may be quite scarce in the natural diet, whereas it is unlikely to be a limiting factor during breeding, when plasma carotenoid levels are 10–50 μg mL^{-1} (Table 3). Using a median of 30 μg mL^{-1}, this is two to five-fold greater than the concentrations in most of the 40 passerine species investigated by Tella *et al*. (2004), including the congeneric blue tit *Parus caeruleus* (3.6 μg mL^{-1}), and superseded only by the heavily pigmented Northern Cardinal *Cardinalis cardinalis* (40 μg mL^{-1}) and European goldfinch *Carduelis carduelis* (53 μg mL^{-1}).

With respect to sexual differences, females had slightly lower carotenoid levels than males, although

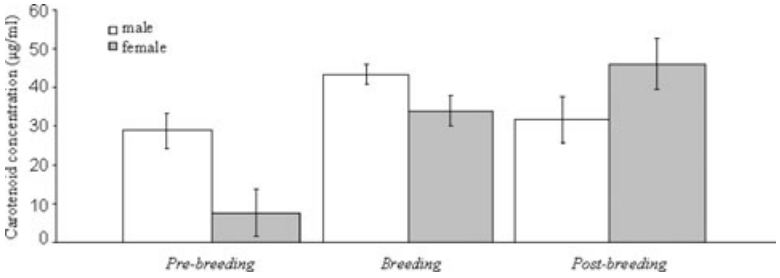

Figure 1. Plasma carotenoid concentration in males and females in different seasons. Presented as least square means ± standard error from model (Table 2).

Table 3. Plasma carotenoid levels in different years, seasons and between different sexes in the great tit

	Prebreeding		During breeding		Postbreeding	
Year	Female	Male	Female	Male	Female	Male
2002	–	–	12.85 ± 3.65	19.53 ± 2.09	–	4.23 ± 0.54
2003	14.40 ± 1.88	15.20 ± 1.93	49.37 ± 3.48	50.63 ± 4.87	–	–
2004	–	25.83 ± 3.24	20.79 ± 2.69	26.22 ± 4.50	30.00 ± 3.23	29.68 ± 5.47

Mean ± standard error total carotenoid concentration ($\mu g\ mL^{-1}$).

the sample sizes were not large enough for a significant sexual difference in any given season (Table 2). However, the significant interaction showed that sexes differed in their patterns of seasonal change (Table 2; Fig. 1), with females being more carotenoid-deprived than males before leaf emergence and then showing increasing levels (Fig. 1), whereas males had less variable levels (Fig. 1). The prebreeding difference is possibly due to female carotenoid allocation to egg yolk (Hõrak, Surai & Møller, 2002; Blount *et al*., 2004; Biard, Surai & Møller, 2005).

Abiotic environmental factors that influence food availability (Myers, 1998; Verboven, Tinbergen & Verhulst, 2001; Jones, Doran & Holmes, 2003) differ not only between seasons, but also between years, which are the most likely explanation for the notable yearly differences in circulating levels of carotenoids.

Previously, we have also shown that urban great tits exhibit higher levels of oxidative stress during the breeding season, measured as the ratio between oxidized and reduced form of glutathione (Isaksson *et al*., 2005). Under the assumption that carotenoids are used as limiting sorurce of antioxidants, we expected the urban birds to have lower plasma concentrations than rural birds (but see Hartley & Kennedy, 2004). There was, however, no such differences between the habitats, which may suggest, as indicated above, that neither dietary access, nor utilization by the immune system are actual carotenoid constraints in these great tit populations.

In accordance with Hill (1995a), we found a positive relationship between plasma concentration at moult and plumage carotenoid deposition and, in great tits, this effect was independent of sex. Furthermore, plasma carotenoid concentrations during breeding were weakly negatively correlated with plumage concentrations. In house finches, *Carpodacus mexicanus*, Hill (1995a) found a similar negative but not quite significant negative association during breeding. A possible explanation, at least in the present study, is that the 2002 season, from which the negative correlation derives, had particularly low plasma carotenoid levels and that the most depleted individuals were those that had deposited most plumage carotenoids during the preceding moult. This could be either because they were still suffering from the allocation 9 months earlier, or because of a propensity to store pigments (e.g. in the liver) and mobilize them at the time of moult. However, we found no relationship between body condition and plasma carotenoid levels. Nor did body condition influence the carotenoid deposition in feathers, as presumed by honest signalling theory (Hill & Montgomerie, 1994; von Schantz *et al*., 1999; Senar, Figuerola & Domenech, 2003), but because great tits are sexually monomorphic in this respect, the lack of a relationship may not be surprising. There was, however, a highly significant negative effect of urban habitat on body condition, which could be induced by several anthropogenic factors.

In summary, we have shown that plasma carotenoid concentrations are relatively high in wild great tits, especially during breeding with access to foliovorous caterpillars. Concentrations vary substantially between sex, seasons, and years but no difference was detected between urban and rural populations, despite earlier findings that urban great tits are more oxidatively stressed. The sexual differences before breeding are probably related primarily to the high maternal carotenoid deposition in egg yolk. Finally, there was a weak but positive association between plumage pigmentation and plasma levels during moult, suggesting that the yellow coloration may still function as an honest signal of carotenoid status and health.

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