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# Carotenoid pigmentation does not reflect total non-enzymatic antioxidant activity in plasma of adult and nestling great tits, *Parus major*

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**Summary**

**1.** Based on carotenoids' antioxidant and pro-vitamin functions, carotenoid pigmentation, particularly in birds, has been hypothesized to honestly advertize condition and health. As antioxidants, carotenoids protect the body from oxidative damage caused by internal or external stressors (e.g. growth, infection and anthropogenic pollution). However, the relative importance of carotenoids in the avian antioxidant defence has been questioned, and the positive relationship between colouration and health may be a 'red herring'.

**2.** In the present study, we investigate carotenoid-based plumage colouration and circulating carotenoids in relation to plasma non-enzymatic total antioxidant activity (TAA) in adult and nestling great tits (*Parus major*). The study was conducted in urban vs. rural populations, with documented differences in oxidative stress level and plumage pigmentation.

**3.** First, we found that there was no relationship between TAA and plumage pigmentation (carotenoid chroma) or plasma carotenoids. Second, urban environment significantly influenced TAA; old (2+ years) urban birds had higher activity than old rural birds, and also compared to younger (1 year) urban adults. This is likely to be due to the increased demand for plasma antioxidants as a consequence of urban environmental stress. Third, nestlings' TAA showed no difference between urban and rural environments. However, it was highly influenced by brood, but not related to parental levels, therefore most likely due to differences in the nestling environment.

**4.** We suggest that carotenoids are not significant contributors to extra-cellular antioxidant defence in great tits, and that carotenoid pigmentation may be an overrated health indicator in this respect. Honest carotenoid signalling, when present, is thus likely to be mediated by some other constraint, such as nutrition or uptake, or other health-modulating mechanisms.

*Key-words*: oxidative stress, plasma carotenoids, spectral reflectance, TAA, urban environmental stress

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## Introduction

It has been suggested that carotenoid-based plumage pigmentation in birds honestly reveals individual quality (genetic or phenotypic), because limited access to carotenoids in the diet makes them indicative of foraging skills, or because the carotenoid content in the plumage reflects immuno-competence or antioxidant status (e.g. Endler 1980; Hill 1990; Andersson 1994a; Lozano 1994; von Schantz *et al*. 1999). Dietary supplementation of carotenoids in birds has repeatedly been shown to have positive effects on both carotenoid pigmentation (i.e. plumage and bare-parts) and immune response (Blount *et al*. 2003a; McGraw & Ardia 2004; Alonso-Alvarez *et al*. 2004), suggesting that an allocation conflict between the two could occur. The links between fitness and antioxidant defences have been relatively less studied, but plasma based antioxidant levels have been linked to breeding performance and life span (Blount *et al*. 2003b, 2006; Alonso-Alvarez *et al*. 2006). However, the importance of carotenoids in the multifaceted



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avian antioxidant defence is poorly understood (e.g. Britton 1995; Surai 2002; El-Agamey *et al*. 2004). In fact, their importance *in vivo* is debated and the association between carotenoid pigmentation and health has therefore been suggested to be a 'red herring' (see Hartley & Kennedy 2004; Bertrand *et al*. 2006). For example, instead of acting as antioxidants, plasma carotenoids might correlate with other free radical scavengers, such as other non-enzymatic plasma antioxidants (e.g. α-tocopherol, albumin, bilirubin, ascorbic acid, uric acid and reduced glutathione) or antioxidant enzymes (e.g. glutathione peroxidase, glutathione S-transferase and catalase) (see Surai 2002), leading to a relationship between carotenoid-based traits and other compounds that may be better free radical scavengers than carotenoids (e.g. Biard *et al*. 2006, see also Hõrak *et al*. 2004). However, even though a positive relationship between plumage pigmentation and plasma antioxidants are present, it is also possible that there is a negative relationship between other antioxidants and plasma carotenoids, due to carotenoid allocation to plumage.

Previously, we have shown that great tits (*Parus major*) living in an urban environment had higher levels of oxidized to reduced glutathione (Isaksson *et al*. 2005), an important intra-cellular antioxidant system (Halliwell & Gutteridge 2002), which was accompanied by a less chromatic yellow breast plumage (Isaksson *et al*. 2005, see also Hõrak *et al*. 2000). In the present study, we address the issue of how important the contribution of carotenoids is to the efficacy of the non-enzymatic component of the antioxidant defence system in bird plasma. We investigate, for the first time, the relationships between plumage pigmentation, plasma carotenoids, and total antioxidant activity (TAA) in urban and rural (high vs. low air pollution level) populations of adult and nestling great tits.

The difference between urban and rural environments includes several human-induced factors, such as diet, artificial light and sounds, and air pollution. However, urban air pollution in particular (e.g. nitrogen oxides and particulate matter) is known to increase the internal levels of reactive oxygen or nitrogen species (ROS or RNS) (e.g. Risom *et al*. 2005). These can cause damage to biologically important molecules if not scavenged or quenched by antioxidants (e.g. reviewed in Surai 2002). Thus, the need for high antioxidant activity will be greater in the urban environment. Since the antioxidant system is very complex and multifaceted, overall measures of antioxidant activity have an advantage over more specific measures (e.g. of single components such as glutathione) and will thus provide a better and more general indication of individual stress. Here we use the measure referred to as TAA (i.e. total antioxidant activity). TAA captures all non-enzymatic antioxidants (see above) circulating in the plasma along with its capacity to trap free radicals (Re *et al*. 1999). The endogenously produced antioxidants (e.g. uric acid, ascorbic acid (vitamin C), bilirubin and albumin) have the highest impact on TAA, and will be up-regulated as a response to oxidative stress (e.g. Pérez-Campo *et al*. 1994). In humans, however, around 15% of the variation in TAA is ascribed to dietary antioxidants (e.g. carotenoids and α-tocopherol) (Miller *et al*. 1993; Re *et al*. 1999).

The main questions that we address are: Does carotenoid-based plumage colouration directly reflect the total of free radical scavenging capacity of the plasma? Do plasma carotenoids correlate with the total plasma antioxidant capacity? Finally, does more oxidatively stressful urban environment elevate the TAA?

## Materials and methods

### STUDY SPECIES AND DATA COLLECTION

The great tit (*P. major*) is a common resident bird of woodlands, gardens and city parks in Sweden. Our urban study areas (Slottsskogen and Änggårdsbergen) are located in the city of Göteborg, and the rural areas (Gräppås, Högås and Råön) are located ca. 50 km southwest of Göteborg. The two environments have documented differences in air pollution levels (Kindbom *et al*. 2001), but the habitats (i.e. deciduous forest) are similar as regards vegetation, dominated by oak: *Quercus* sp., birch: *Betula* sp. and pine: *Pinus* sp.

Nestlings were measured when 11–16 days old ($n = 49$, 88% on day 13), a few days before fledging (approximately occurring at 18 days of age). The slight age variation at this stage did not influence skeletal body size (tarsus length) (ANOVA: $F_{1,259} = 0{\cdot}09$, $P = 0{\cdot}769$). Great tit nestlings were weighed with a Pesola spring balance (± 0·1 g), and tarsus length was measured with a sliding caliper (± 0·01 mm), a blood sample was taken and plumage colour was measured (see below). Adults were caught (in nest box traps) and measured on the same day as nestlings. Body condition was calculated as log mass/(3 × log tars) (Andersson 1994b). In total, we handled 261 nestlings from 49 broods, and 39 females and 26 males. Mean brood size was 5·72 ± 0·21. However, all members of the same family could not be sampled with respect to all target traits (e.g. due to failed capture or blood samples); sample sizes were therefore reduced for some analyses (see Results section).

### PLUMAGE PIGMENTATION AND MEASUREMENTS

The yellow breast plumage of both nestlings and adults is based on the dietary carotenoids, lutein and zeaxanthin, which are also the major pigments present in plasma (Partali *et al*. 1987; Isaksson & Andersson 2007). Adults (yearlings (i.e. 1-year breeders) and older (2+ years)) were classified with plumage characteristics (Svensson 1992).

We took three reflectance scans (removing the probe between each) from the yellow flank plumage, just

below the white bar on the folded right wing, using a S2000 spectrometer system (Ocean Optics Inc, Dunedin, USA) and C-SPEC software (Ancal Inc, Las Vegas, USA). Before each individual was measured, a dark current and a white reference scan (WS-2, > 98% reflectance within wavelengths 300–800 nm) were obtained.

From the raw spectral reflectance data, we computed and averaged for each individual, 'carotenoid chroma' $(R_{700}–R_{450})/R_{average}$, which is the best indicator of carotenoid content in feathers (Andersson & Prager 2006; Isaksson *et al*. unpublished), spectral location ('hue'), which was estimated as $\lambda(R_{50})$, the wavelength at which the reflectance is halfway between its minimum ($R_{min}$) and its maximum ($R_{max}$), and spectral intensity ('brightness'), which was estimated as the total reflectance ($R_{tot}$) from 400 to 700 nm (for further details see Andersson & Prager 2006).

## ANTIOXIDANT ANALYSIS

### *Blood samples*

We used a heparinized syringe to draw *c.* 200 µL blood from the neck vein. All blood samples were kept on ice until later the same day when the plasma was separated by centrifugation at 1800 r.p.m. for 10 min, and immediately frozen at –80 °C.

### *Total antioxidant activity (TAA)*

Total antioxidant activity (TAA) was measured in 10 µL plasma using a decolourization assay (Re *et al*. 1999). This technique accurately measure the total non-enzymatic antioxidant concentration and its antioxidant capacity by allowing a complete reaction between the antioxidants and the radical cation 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^+$) (Re *et al*. 1999) and is more robust than similar techniques that disrupt the reaction after a specific time (e.g. Miller *et al*. 1993). The colour reduction (the lower absorptance the higher TAA) is measured spectrophotometrically at 734 nm, and compared to an internal standard (Trolox, a Vitamin E analogue). Results are presented as µmol Trolox equivalents per mL plasma. Samples were run singly, but this kind of assay is widely used in studies on human plasma, has good repeatability, and correlates well with other measures of antioxidant activity (e.g. Bompadre *et al*. 2004; Thaipong *et al*. 2006).

### *Carotenoid extraction and high performance liquid chromatography (HPLC)*

The day before the analysis, plasma samples were thawed and 20 µL was removed, mixed with 380 µL acetone and frozen over night at –80 °C. Next day, the samples were defrosted, and the liquid phase was filtered through a 0·2 µm syringe filter (GHP Acrodisc 13 mm, Pall Gelman Sciences Inc. Ann Arbor, USA) and evaporated in a vacuum centrifuge (Savant DNA 120, Holbrook, Arizona, USA). Following resuspension in 100 µL of the HPLC mobile phase (70 : 30 acetonitrile : methanol, v/v), 20–40 µL of the sample was injected into a RP-18 column (ODS-AL, 150 × 4·0 mm i.d., YMC Europe GmbH, Schermbeck, Germany), fitted on a ThermoFinnigan (San Jose, USA) HPLC system with PS4000 ternary pump, AS3000 autosampler and UV6000 diode-array UV/VIS detector. Column temperature was maintained at 30 °C. A 2D (at 450 nm) and 3D (300–700 nm) chromatograms were obtained and analyzed with CHROMQUEST 4·0 software (ThermoFinnigan, San Jose, CA, USA). Carotenoid concentrations are calculated and presented as µg/mL plasma, *c.* 87% of plasma carotenoids are lutein and 9% zeaxanthin.

## DATA HANDLING AND STATISTICAL ANALYSIS

We separated the data into the two main environments, urban (two study sites) and rural (three study sites). To identify factors explaining the variance in TAA and plasma carotenoids we used general linear mixed models (GLMM). For nestlings, environment (urban vs. rural) was used as a fixed factor and brood was initially included as a random factor, nested within environment, condition (standardized with respect to nestling age) was included as covariate, and its interaction with the fixed factor was included in the initial model (Table 1). For adults, environment was included as a fixed factor, together with age and sex. Brood size and condition were included as covariates in the initial models (Table 2). We started with the full model and

**Table 1.** Full model for TAA in nestlings

| | d.f. | *F* | *P*-value |
|---|---|---|---|
| Environment | 1 | 0·007 | 0·934 |
| Family (random) | 46 | 1·445 | 0·045 |
| Condition | 1 | 0·023 | 0·881 |
| Condition × Environment | 1 | 0·536 | 0·465 |

**Table 2.** Full model for TAA in adults

| | *F* | *P*-value | *F** | *P*-value* |
|---|---|---|---|---|
| Environment* | 0·758 | 0·388 | 0·039 | 0·845 |
| Age* | 3·690 | 0·060 | 5·058 | 0·028 |
| Sex | 0·128 | 0·722 | – | – |
| Broodsize | 0·593 | 0·445 | – | – |
| Condition* | 0·303 | 0·584 | – | – |
| Age × Environment* | 4·300 | 0·043 | 3·699 | 0·059 |
| Sex × Environment | 2·413 | 0·126 | – | – |
| Brood size × Environment | 0·002 | 0·968 | – | – |
| Condition × Environment | 1·497 | 0·227 | – | – |

All factors in initial model, d.f. = 1, whole model error = 53, $n = 62$.
*All factors in final model, d.f. = 1, model error = 59, $n = 62$.

used backward elimination at $P > 0{\cdot}25$ (see Quinn & Keough 2002). Full, initial, models are provided in Tables 1 and 2. Simple regressions and mean values per brood were used in all other analysis. All data were checked for normality and homogeneity of variances. *Post hoc* analyses of power were not appropriate for the present study. Two outliers (more than three standard deviations from the mean) from different families were excluded from analysis with TAA. Means are presented with ± SE. All analysis were performed in JMP 5·1 (SAS Institute Inc., 2003, Cary, NC, USA).

## Results

### NESTLINGS

Mean TAA for nestlings was 32·92 ± 0·56 µmol Trolox equivalent/mL plasma. There was no relationship between TAA and nestling plumage colouration (carotenoid chroma: $F_{1,46} = 0{\cdot}066$, $P = 0{\cdot}798$, see Fig. 1a; hue: $F_{1,46} = 0{\cdot}230$, $P = 0{\cdot}634$; brightness: $F_{1,46} = 0{\cdot}382$, $P = 0{\cdot}130$) or plasma carotenoids (total concentration: $F_{1,45} = 1{\cdot}36$, $P = 0{\cdot}249$, see Fig. 1b, lutein: $F_{1,45} = 1{\cdot}156$, $P = 0{\cdot}288$, zeaxanthin: $F_{1,45} = 2{\cdot}186$, $P = 0{\cdot}146$). TAA differed among broods (GLMM, brood, $F_{48,201} = 1{\cdot}56$, $P = 0{\cdot}019$), but there was no effect of urban vs. rural environment or nestling condition (all $P > 0{\cdot}25$) (see Table 1). Brood size did not explain the brood effect on TAA ($F_{1,35} = 0{\cdot}019$, $P = 0{\cdot}892$). Moreover, nestling TAA was not associated with parental TAA (mother, $F_{1,34} = 2{\cdot}69$, $P = 0{\cdot}110$; father, $F_{1,19} = 1{\cdot}68$, $P = 0{\cdot}211$).

Mean total carotenoid concentrations for nestlings was 45·15 ± 1·8 µg/mL plasma. As with TAA, plasma carotenoid concentration differed significantly among broods, but again environment had no effect (GLMM, brood, $F_{46,168} = 5{\cdot}69$, $P < 0{\cdot}0001$; environment, $F_{1,46} = 2{\cdot}87$, $P = 0{\cdot}097$). The carotenoid concentration of nestling plasma was not associated with parental plasma carotenoid concentration (mother, $F_{1,30} = 0{\cdot}01$, $P = 0{\cdot}919$; father, $F_{1,19} = 1{\cdot}98$, $P = 0{\cdot}176$). Finally, there was a significant positive correlation between mean nestling plumage carotenoid chroma and mean plasma carotenoid concentration ($R^2 = 0{\cdot}21$, $F_{1,45} = 11{\cdot}77$, $P = 0{\cdot}0013$).

### ADULTS

Mean total antioxidant activities and mean carotenoid concentration for adults are shown in Figs 2 and 3, respectively. Similarly to nestlings, there was no relationship between TAA and carotenoid chroma (TAA: $F_{1,48} = 0{\cdot}667$, $P = 0{\cdot}418$; sex: $F_{1,48} = 0{\cdot}131$, $P = 0{\cdot}719$, see Fig. 1a), brightness (TAA: $F_{1,48} = 0{\cdot}001$, $P = 0{\cdot}983$; sex: $F_{1,48} = 3{\cdot}734$, $P = 0{\cdot}059$) or hue (TAA: $F_{1,46} = 3{\cdot}300$, $P = 0{\cdot}076$; sex: $F_{1,46} = 5{\cdot}850$, $P = 0{\cdot}020$; environment: $F_{1,46} = 0{\cdot}044$, $P = 0{\cdot}835$; environment × sex: $F_{1,46} = 5{\cdot}632$, $P = 0{\cdot}022$). Carotenoid chroma was negatively correlated with plasma carotenoids ($F_{1,41} = 3{\cdot}988$, $P = 0{\cdot}0525$).

Moreover, TAA in plasma was not associated with carotenoid concentration (total concentration: $F_{1,50} = 0{\cdot}049$, $P = 0{\cdot}826$, see Fig. 1b, lutein: $F_{1,50} = 0{\cdot}025$,

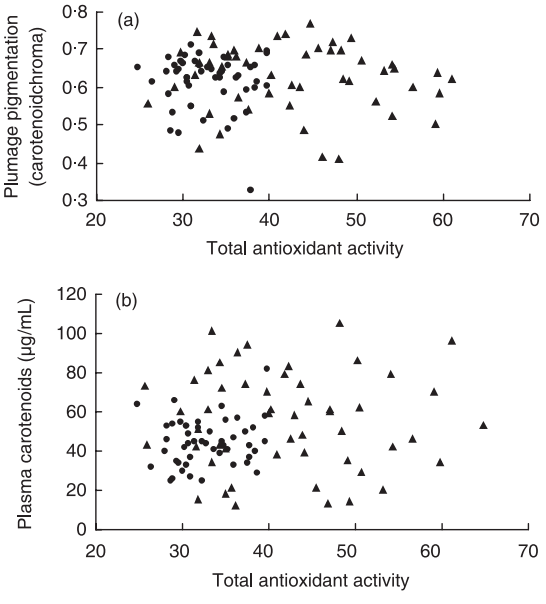


**Fig. 1.** Relationship between total antioxidant activity (TAA, µmol of Trolox equivalent/mL) and (a) yellow plumage pigmentation; and (b) plasma carotenoids (µg/mL) in adult (triangles) and nestling (circles) great tits. Nestlings are presented as mean per brood.

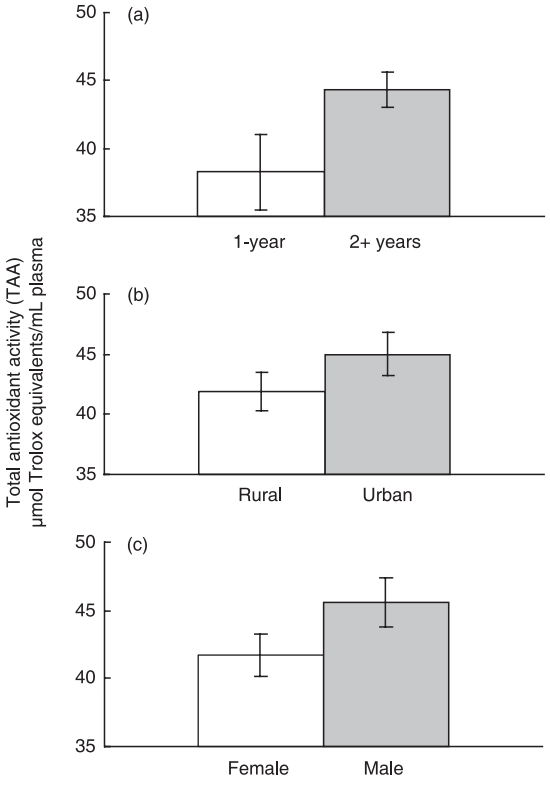


**Fig. 2.** Total antioxidant activity (TAA) presented as µmol of Trolox equivalent/mL plasma in (a) young and older; (b) rural and urban; and (c) female and male adult great tits. Mean ± SE are presented.

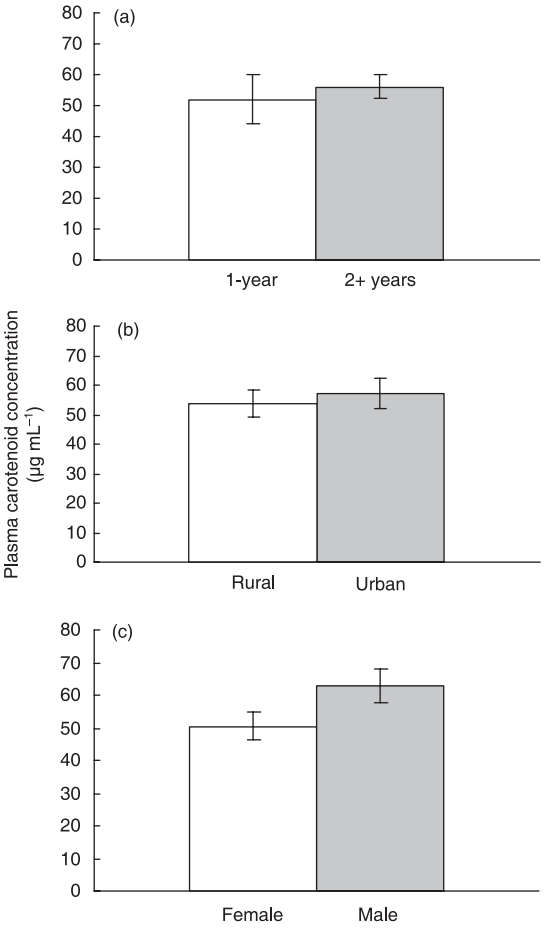


**Fig. 3.** Total carotenoid concentration in plasma, presented as μg/mL plasma in (a) young and older; (b) rural and urban; and (c) female and male adult great tits. Mean ± SE are presented.

$P = 0{\cdot}874$, zeaxanthin: $F_{1,50} = 0{\cdot}749$, $P = 0{\cdot}391$). Neither was there any general effect of urban environment on adult TAA level (environment: $F_{1,59} = 0{\cdot}039$, $P = 0{\cdot}845$, age: $F_{1,59} = 5{\cdot}058$, $P = 0{\cdot}028$, environment × age: $F_{1,59} = 3{\cdot}699$, $P = 0{\cdot}059$, for full model see Table 2). However, the border-line significant interaction revealed that old urban adults had higher activity compared to similar aged rural adults and young urban adults, whereas there was no age difference in activity between old and young rural adults. Adult sex was excluded from the model ($P > 0{\cdot}25$, Table 2). Moreover, adding nestlings to the model showed a significant increase in TAA with age (LSM, nestlings: $3{\cdot}485 \pm 0{\cdot}026$, yearlings: $3{\cdot}630 \pm 0{\cdot}054$, older: $3{\cdot}769 \pm 0{\cdot}025$, Two-way ANOVA; age: $F_{2,106} = 33{\cdot}566$, $P < 0{\cdot}001$; environment: $F_{1,106} = 0{\cdot}061$, $P = 0{\cdot}806$; age × environment: $F_{2,106} = 3{\cdot}253$, $P = 0{\cdot}043$).

## Discussion

Less than a decade ago, von Schantz *et al*. (1999) suggested that carotenoids mediate honest quality signalling via a trade-off between ornamental pigmentation and internal antioxidant capacity. Although the roles of carotenoids in both health and colouration (e.g. Lozano 1994) already were well established at that time, their specific importance as antioxidants *in vivo* is still debated (see Hartley & Kennedy 2004) and rarely empirically explored. For example, it is not known whether carotenoids contribute directly to the antioxidant defence, or if they are only indirectly related due to correlations with other, more important antioxidant systems, such as uric acid and α-tocopherol (Hartley & Kennedy 2004). This study refutes both alternatives, since: (i) carotenoid pigmentation did not reflect individual non-enzymatic antioxidant activity (TAA); and (ii) there was no correlation between circulating (plasma) carotenoids and TAA.

TAA summarizes the activity and concentration of all the different dietary antioxidants, (e.g. carotenoids and α-tocopherol) as well as endogenously generated non-enzymatic antioxidants present in the plasma (e.g. glutathione, vitamin C and uric acid). Thus, it is an accurate measure of oxidative stress level and should therefore be negatively correlated with carotenoid pigmentation under the hypothesis that plumage pigmentation indicates health (e.g. Lozano 1994; von Schantz *et al*. 1999). However, there was no relationship between TAA and carotenoid chroma (the colorimetric estimate that most accurately reflects carotenoid content of feathers, Andersson & Prager 2006), neither in nestlings, nor in adults. A problem with these conclusions, for adults, is that the measurements were obtained in spring and thereby, there may have been a relationship between pigmentation and TAA during moult (i.e. preceding autumn). Even so, however, it is still a valid conclusion that plumage carotenoid colouration is not a reliable signal of current oxidative stress, and during the time when such qualities are most important to assess.

Curiously, while carotenoid chroma (the primary measure of pigment content in unsaturated carotenoid colours; Andersson & Prager 2006) was highly unrelated to TAA, hue ($\lambda R_{50}$) showed a trend to a negative association with TAA. However, although hue normally also is positively related to carotenoid concentration, this does not seem to be the case in the great tit (Isaksson *et al*. unpublished). In a detailed study of reflectance variation and sexual dimorphism in great tits, we show that females have both lower brightness (probably due to more melanin) and a slightly more longwave hue, but that this is unrelated to the carotenoid content (Isaksson *et al*. unpublished). In the present study, the five most longwave hues had also the lowest TAA values and were all females. This tendency of lower TAA in females with more longwaved hue is interesting in itself and requires further studies, but it remains unlikely that carotenoid pigmentation is the mechanism. Regarding plasma carotenoids, they accurately reflected plumage pigmentation for nestlings, but for adults there was a negative correlation between the two, suggesting a delayed immune cost of carotenoid incorporation (see also Hill 1995; Isaksson *et al*. in press).

Importantly, there was no relationship in nestlings or adults between TAA and circulating concentrations of carotenoids. The lack of relationship may suggest that carotenoids either are: (i) insignificant contributors to TAA (i.e. not active as free radical scavengers in plasma); or (ii) that the levels of other (synthesized) antioxidants are adjusted relative to carotenoid levels to maintain antioxidant capacity. However, since the individual variation is large the second hypothesis is unlikely. But, either way our results suggest that neither pigmentation nor plasma carotenoids provide direct information on variation in TAA. A recent study on zebra finches (*Taeniopygia guttata*) found a positive relationship between the change of plasma carotenoids (i.e. before vs. after carotenoid supplementation) and resistance to oxidative stress (measured as resistance of red blood cells to a controlled free radical attack) and also to the sexually selected red beak (Alonso-Alvarez *et al*. 2004). The actual carotenoid availability was, however, not significantly related to their measure of resistance to oxidative stress. Similar results were found in Eurasian kestrels (*Falco tinnunculus*), in which carotenoid concentration in plasma was unrelated to oxidants, antioxidants and degree of oxidative stress (Costantini *et al*. 2006), suggesting that carotenoids are most likely not used as antioxidants in the plasma.

Our study was conducted in urban vs. rural populations of great tits. Individuals living in an urban environment with high anthropogenic pollution, should generally experience higher levels of oxidative stressors (e.g. Risom *et al*. 2005), and hence antioxidant activity should increase to counter oxidative damages. In the present study, old urban living great tits had up-regulated antioxidant activity in relation to similar aged rural birds, and also in relation to younger adults breeding in the urban environment (see also Isaksson *et al*. 2005). The age effect may be a response to a longer exposure time of urban pollutants, in particular since there was no difference in TAA between the ages in the rural environment. The lack of environmental effect on nestling TAA is probably an effect of a shorter time of exposure, along with a not yet fully developed defence system (Pastoret *et al*. 1998). Moreover, the enzymatic antioxidant defence is probably even more likely to respond to environmental change or stress than the non-enzymatic defence system. Unfortunately, to date this has not been investigated in urban vs. rural populations of birds.

As expected, we found strong effects of brood on nestling TAA and plasma carotenoids. Both TAA and plasma carotenoids are influenced by the diet (i.e. quantity and quality) and nestlings rely on the parents for food delivery and, consequently, post-hatching parental effects are important for antioxidant defence (Costantini & Dell'Omo 2006; Isaksson & Andersson 2007). For example, in the Eurasian kestrel, Costantini & Dell'Omo (2006) showed that rearing environment was responsible for 52·8% of the variation in serum antioxidant barrier (OXY). Indeed, both TAA and plasma carotenoids were unrelated to parental plasma antioxidant activity, suggesting that most variation arises from variation in rearing conditions, although genetic and epigenetic inheritance of both assimilation and synthesis of the non-enzymatic antioxidant defences can influence inter-family variation (Isaksson *et al*. 2006).

To summarize, our results suggest that: (i) carotenoid-based plumage colouration does not reflect total non-enzymatic antioxidant activity of the plasma (i.e. stress level); (ii) plasma concentration of carotenoids is not related to plasma TAA, and finally; (iii) aging in an urban environment significantly increases the antioxidant activity compared to aging in a rural environment. These results suggest that carotenoids most likely do not contribute to the extra-cellular antioxidant defence, and signal content, if any, are mediated through other carotenoid-mediated functions.

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