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# Antioxidant protection and plasma carotenoids of incubating great tits (*Parus major* L.) in relation to health state and breeding conditions

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**Abstract**

Carotenoids are biologically active pigments, which are important for animals due to their dual role in health maintenance and ornamental signalling. In adult birds, immunostimulatory properties of carotenoids have been repeatedly demonstrated while much less is known about the importance of carotenoids as antioxidants. We studied the relationships between plasma carotenoid levels, as well as total antioxidant protection, and various hemato-serological health state indices in female great tits (*Parus major* L.), incubating their second clutches in two contrasting (coniferous and deciduous) habitats in southwest Estonia. To manipulate reproductive effort, four eggs were removed from half of the clutches during laying to stimulate females to lay additional eggs. However, egg removal had no effect on the final number of eggs laid. Plasma carotenoid levels increased seasonally in parallel with caterpillar food availability. However, no between-habitat differences in carotenoid levels, total antioxidant capacity, or indices of health state could be found despite the apparently better feeding conditions in the coniferous habitat. No correlation was detected between plasma carotenoid levels and measures of total antioxidant capacity, which suggests that at least for the adult birds feeding on naturally carotenoid-rich diet, antioxidant function of carotenoids is not of primary importance. A strong non-linear association between the measures of antioxidant protection and leukocytic markers of inflammation was found, which suggests that measures of total antioxidant capacity deserve further attention in ecophysiological studies as potential indicators of immunopathology.


*Keywords:* Antioxidant protection; Carotenoids; Caterpillar abundance; Habitat heterogeneity; Heterophils; *H*/*L* ratio; *Parus major*; Seasonality; Second broods

## 1. Introduction

Reproduction in birds demands considerable metabolic investment (e.g., Drent and Daan, 1980), which can be associated with increased production of reactive oxygen species, ROS (Alonso-Alvarez et al., 2004a; Wiersma et al., 2004; Bertrand et al., 2006). ROS are generally unstable and very reactive with a potential to damage nucleic acids, proteins, and lipids (Halliwell and Gutteridge, 1999). To control and neutralise ROS, organisms evolved defence systems based on antioxidants, which deactivate ROS by donating missing electrons. Endogenous antioxidants (uric acid, bilirubin, albumin, and enzymes like catalase, superoxide dismutase, glutathione peroxidase) are synthesized by an organism and exogenous antioxidants such as fat-soluble vitamins E and A, and carotenoids (in many passerines also ascorbic acid) must be obtained from food. Of all these antioxidants, avian ecologists have focussed their primary attention on carotenoids because these are frequently used in integumentary sexual signals and also possess immunostimulatory properties (Lozano, 1994; von Schantz et al., 1999; Møller et al., 2000; McGraw, 2006). Under this view, carotenoid-based ornaments enable individuals to signal their past and/or current health state: carotenoids can be allocated to signalling only if and when they are not needed for immunomodulation at the same time.

Compared to their role in signalling and immunity, the antioxidant function of carotenoids has remained much more poorly understood, even in traditional mammal models (El-Agamey et al., 2004). The situation is even more complicated with birds—given that most avian species live longer than similar-sized mammals despite their higher metabolic rates, the birds are supposed to have evolved unique protective mechanisms against the oxidative damage (Perez-Campo et al., 1998). In birds the protective effect of maternally transferred carotenoids to

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embryos and hatchlings is well established (Surai, 2002; McGraw et al., 2005), but only two studies (Alonso-Alvarez et al., 2004b; Costantini et al., 2006) have measured the relationships between carotenoids and general antioxidant defences in nestlings or adult birds.

Here we address the question about the role of carotenoids in modulation of antioxidant protection in female great tits, incubating their second clutches in two contrasting habitats in southwest Estonia. Great tit is a small (ca. 20 g) passerine, breeding in the forests of most of the Palearctic region (Gosler, 1993). The yellow ventral plumage coloration of great tits is based on lutein and zeaxanthin (Partali et al., 1987; Stradi, 1998), two carotenoids that are particularly abundant in lepidopteran larvae (Partali et al., 1987) that are the main source of great tits food during the breeding season (Partali et al., 1987; Naef-Daenzer et al., 2000). Birds in our study area breed in two contrasting habitats (deciduous and coniferous), where physiological condition of adults and reproductive parameters differ considerably (Mägi and Mänd, 2004; Mänd et al., 2005; Kilgas et al., 2006a). The results of an unpublished pilot study suggested that during the second broods, caterpillar food is especially abundant in coniferous forests where the clutches are also larger and nestlings heavier than in the deciduous forests at that period (Mägi and Mänd, 2004). We therefore expected the physiological condition of the females during the second breeding attempt to be better in coniferous habitat than in the deciduous habitat. To manipulate reproductive effort, four eggs were removed from the half of the clutches during laying to stimulate females to lay additional eggs (e.g., Visser and Lessells, 2001).

Specifically, we addressed the following questions. Under the hypothesis that increased egg-laying effort leads to deterioration of antioxidant protection and general health state (Blount et al., 2004; Williams, 2005), we predicted that females induced to lay extra eggs reveal lower levels of antioxidant protection, carotenoids, and indices of nutritional state as compared to the un-manipulated females. We also predicted that if dietary carotenoids have any important antioxidant function in incubating birds, then their plasma concentrations should positively correlate with plasma total antioxidant capacity. Additionally, we tested whether plasma carotenoid levels differ between habitat types, expecting higher concentrations in coniferous habitat with presumably higher caterpillar food supply at that time. On the same grounds, we predicted parallel seasonal trends in caterpillar abundance and plasma carotenoid levels. Finally, we asked whether markers of antioxidant protection and plasma carotenoid levels correlate with leukocytic health state indices, predicting negative correlations with markers of inflammation and stress, such as heterophile count and heterophile/lymphocyte (*H*/*L*) ratio. We expected negative correlations between markers of antioxidant protection and heterophile hemoconcentration because these inflammatory cells release free radicals which might deplete the antioxidant capacity (e.g., Ames et al. 1993; Kogut et al., 2002; Costantini and Dell'Omo, 2006).

## 2. Materials and methods

The study was conducted near Kilingi Nõmme (58°7′N, 25°5′E), southwest Estonia, in June–July 2005, when great tits were laying and incubating their second clutches. Various forest habitats in the 50 $km^2$ study area were classified as deciduous and coniferous (see Mänd et al., 2005, for details). Deciduous woods occur mainly as isolated patches in agricultural landscape or as 250–500-m-wide galleries along roads and stream valleys. They grow on fertile soils and have rich understory. The most common tree species are grey alder (*Alnus incana*) and silver birch (*Betula pendula*). The coniferous woods are continuous managed pine forests on different nutrient-poor sandy soil or, in the lower parts of the terrain, on the peat soils. The dominant species is Scots pine (*Pinus sylvestris*) that sometimes occurs in mixed stands with Norway spruce (*Picea abies*) on sandy soil, or with downy birch (*Betula pubescens*) on peat soil. Great tits bred in wooden nest boxes that were fixed on tree trunks at a height of 1.5–2.0 m. Distance between neighbouring nest boxes was 50–60 m. All occupied nest boxes were inspected daily to determine each egg's laying date, completion of the clutch and starting of incubation. All the eggs in each clutch were consecutively numbered.

### 2.1. Experiment

Within habitats, dyads of randomly selected breeding females with the same egg-laying initiation date were formed. One clutch of the dyad was assigned as control and the other as experimental clutch. On the third and fifth day of egg laying two eggs were removed from the experimental clutches (i.e., total of four eggs). To standardize incubation effort between experimental and control clutches, clutch sizes of dyad members were set equal by adding the appropriate number of eggs to the smaller clutch on the second day of incubation. The substitution eggs originated either from the female's own clutch (the earlier removed ones) or came from donor clutches that were completed on the same date.

### 2.2. General procedures

On the fifth day of incubation female birds were captured from nest boxes. Birds were weighed using a Pesola spring balance with a precision of 0.1 g and the tarsus length was measured with a sliding caliper with a precision of 0.1 mm. Blood samples (300–450 μl) were collected from tarsal or brachial vein. Plasma was separated from erythrocytes with 10 min centrifugation at 10,000 rpm (10,621 g) and stored at −20 °C for 2 weeks and at −70 °C for about 1 month until analysed. All spectrophotometric analyses were performed with Tecan microplate reader (Model Sunrise; Tecan Austria GmbH, Grödig/Salzburg, Austria).

### 2.3. Leukocyte counts

High leukocyte counts can be interpreted to reflect individual's current investment into immune defence (e.g., Nunn et al., 2000) and/or ongoing inflammatory processes (e.g., Jaensch and Clark, 2004). We concentrated on the two most numerous leukocyte types, namely, heterophils and lymphocytes. Heterophils are non-specific phagocytosing cells that

enter the tissues during the inflammatory response. Lymphocytes elicit pathogen-specific immune response. The relative abundance of lymphocytes and heterophils (*H*/*L* ratio) is widely used to estimate stress in poultry (e.g., Maxwell, 1993) and also in wild birds. In great tits, elevated *H*/*L* ratio has been associated with increased reproductive effort (Ots and Hõrak, 1996) and lowered survival probability of breeders (Kilgas et al., 2006b). To assess leukocytic blood parameters, a drop of blood was smeared on microscope slide, which was air-dried, fixed in absolute methanol, and stained with azure–eosin. The proportion of different types of leukocytes was assessed by examining 100 leucocytes under 1000× magnification. Estimates of the total white blood cell count (WBC) were obtained by counting the number of leukocytes per approximately 10,000 erythrocytes. Differential leukocyte counts were obtained by multiplying their proportions with WBC (Ots et al., 1998).

### 2.4. Measures of total antioxidant capacity

We used two methods, based on the capacity of biological fluids to inhibit redox reaction induced by free radicals, for assessment of total antioxidant capacity of plasma. Of these, TAS (Total Antioxidant Status) assay was performed, adapting the commercially available kit (Randox Laboratories, Crumlin, Antrim UK) for small (5 μl) plasma samples. In this assay, the azo-compound ABTS is incubated with metmyoglobin and hydrogen peroxide to produce the radical cation $ABTS^{\bullet+}$. This has a relatively stable blue-green colour, which is measured at 600 nm. Antioxidants in the sample cause suppression of this colour production to a degree, which is proportional to their concentration. The suppression of the absorbance of the $ABTS^{\bullet+}$ radical cation by serum antioxidants was compared with that from a Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid, a water-soluble vitamin E analogue). The results are quantified as mM of Trolox equivalents.

The antioxidant potential (AOP) of plasma was determined using a BIOXYTECH® AOP-490™ assay kit, adapted for small (7.5 μl) plasma samples. This assay is based upon the reduction of $Cu^{2+}$ to $Cu^{+}$ by the combined action of all antioxidants present in the sample. A chromogenic reagent, Bathocuproine (2.9-dimethyl-4.7-diphenyl-1.10-phenanthroline), selectively forms a 2:1 complex with $Cu^{+}$, which has a maximum absorbance at 490 nm. Colour change of the plasma, incubated with reagent containing $Cu^{2+}$ and chromogen during 3 min at room temperature, is measured. A standard of known uric acid concentration was used to create the calibration curve, so the results are quantified in mM uric acid equivalents. Both of those assays are widely used in clinical studies (reviewed in Dotan et al., 2004).

### 2.5. Plasma carotenoids

Concentrations of carotenoids were determined spectrophotometrically using acetone-resistant microtitre plates. 150 μl of acetone was added to 15 μl of plasma and centrifuged for 10 min at 1500×*g*. Absorbance of supernatant was measured at 449 nm, corresponding to the maximum absorbance of lutein in acetone (Zsila et al., 2005). Calibration curves were prepared using lutein (Sigma X 6250) as a standard.

### 2.6. Indices of general condition and nutritional state

To assess general body condition and nutritional state, we measured body mass (at the time of blood samplings) and plasma total protein, albumin, and triglyceride concentrations. High blood triglyceride levels are indicative of a resorptive state during which lipid is formed by the liver and deposited in muscle and adipose tissues (see Kern et al., 2005). Hence, triglyceride concentrations reflect the individual's state of fattening by indicating the amount of lipids absorbed during the few hours before blood sampling (Jenni-Eiermann and Jenni, 1998). Triglyceride concentrations were determined by enzymatic colorimetric test in GPO-PAP Method.

Decrease in total plasma protein concentration accompanies most diseases but is an especially prominent symptom of malnutrition and infections, especially when caused by a decline of plasma albumin level (e.g., Kawai, 1973; Tatum et al., 2000). Total protein and albumin concentrations were determined colorimetrically (Biuret method and BCG-method, respectively). The manufacturer of all three assay kits was Human GBDmbH, (Wiesbaden, Germany). None of the blood parameters recorded in this study was affected by the time of capture (all *P*-values > 0.1).

### 2.7. Frass fall sampling

Caterpillar abundance was monitored by frass-fall method (e.g., Seki and Takano, 1998; Banbura et al., 1999; Visser et al., 2006). Five collecting sites in each habitat were randomly selected, each site containing four collectors (round plastic funnels with 30 cm diameter) placed beneath trees or bushes about 40 cm above the ground. Funnels ended with a 1 × 4 paper coffee machine filter, where the frass accumulated during the collection period. Filters were collected and replaced in every 5 days. Filters with contents were dried at 35–40 °C and stored in plastic bags. Later the frass was separated from the litter, kept at 60 °C for 48 h and weighed with precision of 0.1 mg. For one data-point, the mean of four funnel frass samples in the same collecting place was calculated.

### 2.8. Statistics

All variables except the frass fall amounts and leukocyte counts were normally distributed, so parametric statistical tests could be applied in most of cases. Leukocyte counts of the birds breeding in different habitat types were thus compared by Mann–Whitney *U*-tests and *t*-tests were used for the rest of traits. Dynamics of frass fall was analysed in repeated measures ANOVA with a habitat as a factor, using ln-transformed values. Second-order polynomial regression models were applied when assessing the relationships between TAS and AOP vs. abundance of heterophils and *H*/*L*, because polynomial fit could be obviously detected from the scatterplots and the linear models did not explain the pattern. Residuals from the regression

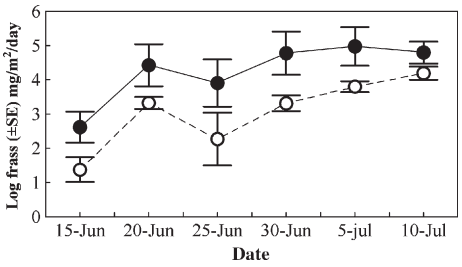


Fig. 1. Average frass fall during the study period. Filled symbols: coniferous habitat, empty symbols: deciduous habitat. Each data point represents average frass fall (summed over 5 days) from five collecting sites. Blood samples from great tits were collected between June 27 and July 10.

analyses did not deviate from normality. α-level was set to 0.05. Analyses were performed with STATISTICA, version 7.1 (StatSoft, Inc., 2005).

## 3. Results

Egg removal had no effect on the final number of eggs laid ($F_{1,44}=1.5$; $P=0.2$) in ANOVA with habitat as a factor ($F_{1,44}=4.1$; $P=0.048$).). Interaction between manipulation and habitat was not significant ($F_{1,43}=0.5$; $P=0.5$). Manipulated females did not differ from controls in any of the hemato-serological or antioxidant parameters either (all $P$-values >0.1).

The amount of frass falls did not differ significantly between the habitats, although there was a marginally not significant tendency to higher frass falls levels in coniferous forest ($F_{1,8}=4.49$, $P=0.067$). The amount of frass fall significantly increased during the season ($F_{5,40}=22.18$, $P<0.0001$; Fig. 1), similarly in both habitats ($F_{5,40}=0.78$, $P=0.57$ for time × habitat interaction in repeated measures ANOVA).

Incubating females in coniferous habitat had higher body mass and tended to lay larger clutches than females in deciduous habitat (Table 1). No significant differences in blood parameters between the two habitats were detected (Table 1).

Plasma carotenoid concentrations of incubating female Great Tits increased with the date of capture (Fig. 2), but no seasonal trends occurred with respect to any other variable (all $P$-values >0.1). There was a strong positive correlation between antioxidant parameters TAS and AOP (Fig. 3). However, no significant correlations between plasma carotenoid levels and AOP ($r=-0.02$, $P=0.9$, $n=37$) or TAS ($r=0.10$, $P=0.5$, $n=37$) emerged.

Both indices of antioxidant protection revealed nonlinear relationships with heterophile concentration: AOP ($F_{1,33}=8.32$, $P=0.007$ for heterophils; $F_{1,33}=7.55$, $P=0.010$ for heterophils

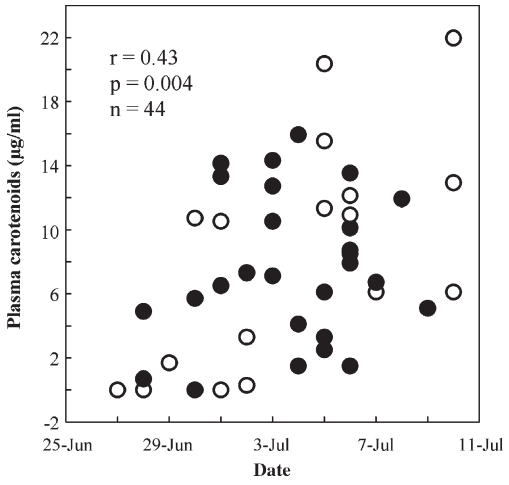


Fig. 2. Plasma carotenoid concentration of female great tits on the fifth day of incubation in relation to date of capture. Filled symbols: coniferous habitat, empty symbols: deciduous habitat.

Table 1
Comparison of reproductive parameters and condition indices of female great tits breeding in coniferous and deciduous habitats

| Trait | Mean±S.D. (*n*) Coniferous habitat | Mean±S.D. (*n*) Deciduous habitat | Statistic | *P* |
|---|---|---|---|---|
| Tarsus length | 19.7±0.7 (32) | 19.3±0.8 (17) | $t=1.32$ | 0.12 |
| Body mass (g)[a] | 20.0±0.8 (32) | 19.2±0.9 (17) | $t=2.75$ | 0.003 |
| Clutch size | 9.5±1.6 (32) | 8.5±1.9 (17) | $t=2.37$ | 0.059 |
| Carotenoids (µg/mL) | 7.6±4.6 (27) | 8.4±7.1 (17) | $t=0.62$ | 0.62 |
| TAS (mM) | 1.8±0.3 (28) | 1.7±0.3 (12) | $t=0.97$ | 0.26 |
| AOP (mM) | 1.7±0.5 (25) | 1.6±0.6 (11) | $t=0.51$ | 0.68 |
| Triglycerides (g/L) | 2.79±4.29 (28) | 1.88±55.8 (12) | $t=0.58$ | 0.47 |
| Total protein (g/L) | 42.2±62.1 (28) | 31.8±4.8 (12) | $t=0.56$ | 0.57 |
| Albumin (g/L) | 22.8±13.4 (28) | 20.8±2.6 (11) | $t=0.42$ | 0.63 |
| WBC/$10^4$ erythrocytes | 36.41±12.96 (32) | 37.59±12.56 (17) | $z=0.52$ | 0.80 |
| Heterophils/$10^4$ erythrocytes | 5.02±4.30 (32) | 5.69±5.00 (17) | $z=0.86$ | 0.39 |
| Lymphocytes/$10^4$ erythrocytes | 31.21±11.11 (32) | 31.21±11.57 (17) | $z=0.17$ | 0.87 |
| *H/L* | 0.16±0.14 (32) | 0.20±0.19 (17) | $z=0.77$ | 0.44 |

Blood samples were taken and body mass recorded on the fifth day of incubation. Leukocyte counts are compared by Mann–Whitney *U*-tests, other parameters by *t*-tests.

[a] Also significant between-habitat difference ($F_{1,\ 47}=5.8$; $P=0.020$) in ANCOVA adjusting for tarsus length.

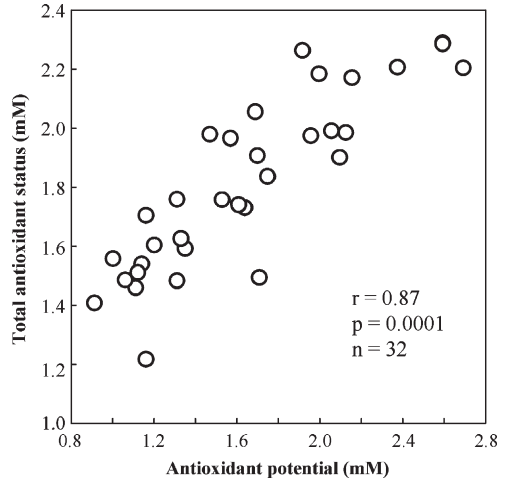


Fig. 3. Correlations between two measures of plasma total antioxidant capacity–total antioxidant status (TAS, mM in Trolox equivalents) and antioxidant potential (AOP, mM in uric acid equivalents).

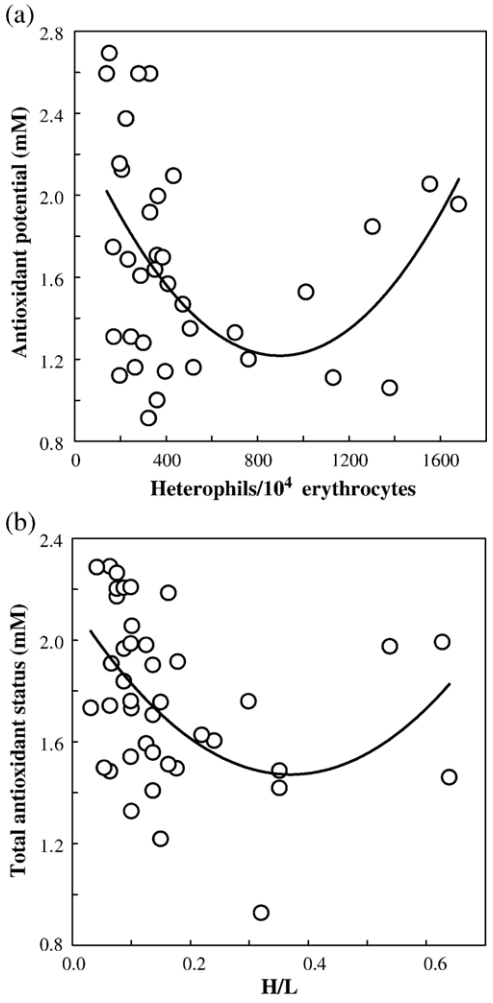


Fig. 4. (a) Relationship between antioxidant potential and heterophile concentration in plasma of female great tits. $N=36$; AOP $=2.345-0.2515\times$(heterophile count) $+0.014\times$(heterophile count)$^2$. Model $R^2=0.20$. (b) Relationship between total antioxidant status and heterophile/lymphocyte ratio ($H/L$) in plasma of female great tits. $N=40$; TAS $=2.143-3.626\times(H/L)+4.8993\times(H/L)^2$. Model $R^2=0.21$.

squared, $R^2=0.20$; Fig. 4); TAS ($F_{1,38}=8.24$, $P=0.007$ for heterophils, $F_{1,38}=6.68$, $P=0.014$ for heterophils squared, $R^2=0.19$). Similar relationships emerged with $H/L$ ratio: AOP ($F_{1,33}=5.37$, $P=0.027$ for $H/L$; $F_{1,33}=4.56$, $P=0.040$ for $H/L^2$, $R^2=0.10$); TAS ($F_{1,38}=8.94$, $P=0.005$ for $H/L$, $F_{1,38}=6.93$, $P=0.012$ for $H/L^2$, $R^2=0.21$).

## 4. Discussion

Frass fall samples revealed increase in caterpillar abundance during the study period in both habitats. Total frass fall index tended to be higher in coniferous woods, similarly to the results of a pilot study in the previous year (unpublished); lack of statistical significance for this difference ($P=0.067$) can be evidently ascribed to the low sample sizes ($n=5$ for both habitats). This conclusion is reinforced by the finding of significantly higher body masses in coniferous habitat, which suggests generally better food availability as compared to deciduous woods. Notably, a previous study in the same area (Kilgas et al., 2006a) has demonstrated a generally better health state of brood rearing great tits in coniferous habitat as compared to their conspecifics breeding in deciduous woodland. However, no such habitat differences emerged in the present study of incubating females.

Our attempt to manipulate egg-laying effort of great tits was unsuccessful as the number of eggs laid by the birds in egg-removal group did not exceed that of the control birds. It is therefore anticipated that physiological variables recorded during the fifth day of incubation did not differ between manipulated and control birds. Lack of response to egg removal was unexpected because similar manipulations in great tits have resulted in laying on average one extra egg (Oppliger et al., 1996; Visser and Lessells, 2001), which hold also for the first breeding attempt in our study populations in 2002 and 2003 (our unpublished data). This discrepancy might be due to the fact that previous successful experiments were conducted in the period of laying first clutches but our experiment was conducted during the second breeding attempt when the birds might have refrained to increase their reproductive effort due to time constraints (Verboven and Verhulst, 1996; Visser et al., 2003).

Similarly to caterpillar abundance, plasma carotenoid levels of incubating female great tits also increased seasonally (Fig. 2), while no between-habitat differences with respect of carotenoids could be detected. Parallel increases in caterpillar abundance and plasma carotenoid levels compare favourably with previous findings that lepidopteran larvae serve as a main source of dietary carotenoids for breeding great tits (Partali et al., 1987; Eeva et al., 1998). On the other hand, in such a case we should have also detected higher plasma carotenoid levels in coniferous habitat, which was not the case. A possible explanation is that the carotenoid content in the needles of coniferous trees is generally lower than that in the leaves of various deciduous trees (Czeczuga, 1987), so that despite the overall higher food availability in conifers, birds get relatively less carotenoids from the same amount of caterpillars eaten. Additionally, similarity of plasma carotenoid levels of birds breeding in deciduous and coniferous woods could be ascribed to the lack of between-habitat differences in health parameters (Table 1).

Under the hypothesis that carotenoids significantly contribute to antioxidative protection, we predicted that individual plasma carotenoid levels correlate positively with measures of total antioxidant capacity. Yet none of such correlations emerged. We are confident that this lack of correlations cannot be ascribed to measurement techniques, because our estimates of total antioxidant capacity, obtained by two different assays were highly correlated (Fig. 3). In line with our results, serum carotenoid concentration did not correlate with measures of antioxidant protection and serum concentration of reactive oxygen metabolites that are a marker of early oxidative damage, in a recent study of kestrel (*Falco tinnunculus*) nestlings (Costantini et al., 2006). Similarly, lutein supplementation to adult captive zebra finches (*Taeniopygia guttata*) had no direct effect on antioxidant barrier (Alonso-Alvarez et al., 2004b). Altogether, these findings suggest that antioxidant properties of carotenoids in birds (except well-established protective effects on embryos and hatchlings) may not appear as important as

previously thought, at least in situations where redox homeostasis is not threatened (see also Hartley and Kennedy, 2004). This view would be also compatible with the results of an extensive meta-analysis of clinical studies of oxidative stress (Dotan et al., 2004), revealing that most antioxidants exhibit a non-monotonic relationship with other criteria of oxidative stress, so that only under severe pathological conditions all the indices of oxidative stress correlate with each other. A recent study suggests that such pathological conditions can be imposed by an immune challenge during growth—immunostimulation of kestrel nestlings with phytohaemagglutinin (PHA) increased the levels of reactive oxygen metabolites and decreased the antioxidant barrier, while the level of circulating carotenoids increased, evidently due to remobilisation from other tissues (Costantini and Dell'Omo, 2006).

Because oxidative stress is involved in pathogenesis of possible infectious and inflammatory disorders (Beckman and Ames, 1998; Halliwell and Gutteridge, 1999), we predicted negative correlations between the indices of total antioxidant capacity and leukocytic markers of inflammation. Such a relationship, although not entirely linear, was indeed found between both our measurements of antioxidant protection and both absolute and relative concentration of heterophils. Heterophils (avian analogues of mammalian neutrophils) are inflammatory cells that bind microbes, internalize them and subsequently kill them by oxidative burst, releasing reactive oxygen and nitrogen intermediates and katabolic enzymes (Ames et al., 1993; Kogut et al., 2002). However, the same free radicals may also cause tissue damages if scavenged insufficiently (e.g., Terron et al., 2003). It is thus possible that the initial decrease in total antioxidant capacity with increasing heterophile hemoconcentration (Fig. 4) reflects diminishing level of antioxidant protection, induced by inflammatory processes which are accompanied by increased traffic of heterophils in the blood stream and excess production of ROS. This explanation would be compatible with the results of Costantini and Dell'Omo (2006), who showed that immune challenge with PHA leads to depletion of total antioxidant defences. (As shown by Hõrak et al., 2000, PHA injection also induces peripheral heterophilia and increases *H*/*L* ratio.) In the current study, however, plasma total antioxidant capacity started to increase again at very high heterophile concentrations (Fig. 4). We cannot therefore exclude the possibility that in some situations increased TAS and AOP levels may reflect compensatory enhancement of antioxidant defences, induced by inflammatory reaction. Such a situation would be analogous to compensatory increase of total antioxidant status subsequent to exercise-induced oxidative stress, as frequently reported in sports medicine (e.g., Vider et al., 2001).

In conclusion, plasma carotenoid levels of incubating great tits increased seasonally in parallel with caterpillar food availability. However, there were no between-habitat differences in carotenoid levels, total antioxidant capacity or indices of health state despite the apparently better feeding conditions in the coniferous habitat. Plasma carotenoid levels were not correlated with measures of total antioxidant capacity, which suggests that at least for the adult birds feeding on naturally carotenoid-rich diet, antioxidant function of carotenoids is not of primary importance. On the other hand, a strong association between the measures of antioxidant protection and leukocytic markers of inflammation was found, which suggests that measures of total antioxidant capacity deserve attention in ecophysiological studies as potential indicators of immunopathology.

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