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Does anthropogenic metal pollution affect carotenoid colouration, antioxidative capacity and physiological condition of great tits (*Parus major*)?

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ABSTRACT

Although several studies have shown that carotenoid-based signals are negatively affected by (metal) pollution, the underlying mechanisms are not well understood. There are two possible, not mutually exclusive, hypotheses to explain the metal-induced fading of carotenoid colouration. Metal pollution could affect oxidative stress levels and/or the diet. We assessed the expression of the yellow breast of adult and nestling great tits (*Parus major*) and related this to physiological parameters in plasma indicative of oxidative stress (total antioxidative capacity) and nutritional condition (albumin, triglyceride, total protein, cholesterol and uric acid concentrations). In four study sites along a metal pollution gradient, both adult and nestling great tits had significantly reduced carotenoid colouration at the most polluted sites. While nestlings' total antioxidative capacity was significantly affected by metal pollution, there was no significant effect on adults' total antioxidative capacity. Both for adult and nestling birds, no clear relation between total antioxidative capacity and carotenoid colouration was found. However, there were significant differences among sites in nutritional parameters, indicating that metal pollution might affect diet composition and quality. We found strong among brood variation in nestlings for all variables (except cholesterol), suggesting that there might be a considerable genetic and/or parental investment factor involved.

1. Introduction

In many animals, females tend to choose the showiest males, assessing them on the expression of their sexual ornaments, which reveals the males' geno- and/or phenotypic quality (Andersson, 1994). In order to be reliable and to prevent cheating, the signal has to encompass a cost (Zahavi, 1975; Johnstone, 1995). One of the most striking examples of sexual ornamentation is the expression of brightly coloured feathers or patches of skin, found mostly in fish and birds. Bold colours can be caused by the microscopic structure of the feathers or skin, or by deposition of pigments such as melanin and carotenoids (Andersson and Prager, 2006). Carotenoids are among the most abundant and bright pigments, responsible for yellow to red colouration. Many studies have investigated which costs carotenoid-based sexual displays could have. One hypothesis states that carotenoids are limited in the environment because they can only be produced by plants. Given the fact that animals have to obtain all of their carotenoids from their diet, carotenoid-based signals could express foraging and carotenoid-uptake efficiency (Olson and Owens, 1998).

Because carotenoids have many different functions (e.g. immunoenhancers, antioxidants, pigments), animals could also be faced with allocation problems. The intensity of the signal could thus reflect the antioxidant status, immunocompetence or parasite load of the individual (Lozano, 1994, von Schantz et al., 1999). Normal metabolism, immune-reactions and parasitic infections are known to increase the production of free radicals (Halliwell and Gutteridge, 2007). Antioxidants are necessary to neutralize these reactive molecules. When there is an imbalance between pro- and antioxidants, oxidative stress can occur, resulting in damage to cells and biomolecules. These antioxidants can be endogenously produced enzymes (e.g. superoxide dismutase, catalase) or molecules (e.g. glutathione) or can be extracted from the diet (e.g. vitamin C, vitamin E, carotenoids). Because carotenoids have antioxidant properties (Krinsky, 2001), it has been proposed that the intensity of a carotenoid-based signal reflects the ability of the individual to cope with oxidative stress (von Schantz et al., 1999). Previous research has studied the hypothesis that oxidative stress could play a key role in the information given by carotenoid-based displays, but the results are not always unambiguous. Alonso-Alvarez et al. (2004) found that higher concentrations of carotenoids in plasma of zebrafinches (*Taeniopygia guttata*) have a positive result on both beak colour and the resistance of red blood cells against oxidative stress. However, in a study on great tits (*Parus major*), Isaksson et al. (2007) found that carotenoid pigmentation does not reflect total non-enzymatic antioxidant activity in plasma (a measurement of oxidative stress) of adults and nestlings. The role of carotenoids as antioxidants *in vivo* was also recently debated by Hartley and Kennedy (2004). They argued that the intensity

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of carotenoid-based signals might not be a direct display of the antioxidant properties of carotenoids but, instead, might be an indicator of uncoloured resources (antioxidants like vitamin C and E) that protect carotenoids from degenerating due to reactive oxygen species (ROS). This is supported by a recent meta-analysis by Costantini and Møller (2008), who showed that carotenoids explain less than 0.002% of the antioxidant capacity of plasma in birds. Therefore, while a decade ago the relationship between carotenoids and oxidative stress seemed quite unambiguous (Lozano, 1994; von Schantz et al., 1999), the role of carotenoids as antioxidants *in vivo* and the information content of carotenoid-based displays recently has become an intensely debated topic (Hartley and Kennedy, 2004).

Some metals, like cadmium and lead, are very toxic and have no known biological function. These toxic effects are partly caused by metal-induced oxidative stress (Ercal et al., 2001). Essential metals, like copper, iron and zinc, can also be toxic when they exceed a maximum concentration (Ercal et al., 2001). In toxic concentrations, these metals can then also generate ROS, which overwhelm the cells antioxidant defenses and result in oxidative stress (Ercal et al., 2001). Previous research studying the effect of metal pollution on carotenoid colouration by Eeva et al. (1998) and Dauwe and Eens (2008) has shown that metal pollution indeed fades the plumage of great tit. These studies did not measure oxidative stress levels and therefore it is not known whether metal-induced oxidative stress was the reason for this fading. Metal pollution could for example also affect food quality and quantity of the diet of great tits living in polluted sites. Individuals living in metal polluted sites could not only have higher levels of oxidative stress, but could also have an altered and lower quality diet affecting their general body condition. This hypothesis is strengthened by studies reporting a reduced amount of suitable food items in polluted areas (Graveland, 1990; Hörnfeldt and Nyholm, 1996) and pollution-related changes in the diet of birds (Eeva et al., 1997, 2005).

In this study, we investigated the effect of metal pollution on both the expression of a carotenoid-based colour and the antioxidant status of the plasma in the great tit for both nestling and adult birds. Because of the (proposed) antioxidant properties of carotenoids, it is possible that animals with carotenoid-based displays living in polluted sites allocate more carotenoids to the antioxidant function and less to pigmentation. To test this hypothesis, we investigated in a field study whether metal pollution negatively affected carotenoid colouration and whether this was related to increased oxidative stress levels. We also examined whether carotenoid colouration is affected by metal-induced differences in diet composition and diet quality of great tits. To test this we compared physiological condition parameters indicative of the nutritional state of organisms among study sites (Jenni-Eiermann and Jenni, 1998). One of the advantages of these measurements is that they provide an insight in the conditions of life of an individual over a longer period of time with only one measurement (Ardia, 2006; Amat et al., 2007). We measured total protein, albumin, uric acid, triglyceride and cholesterol levels in plasma of adult and nestling great tits. These parameters have been used extensively in previous research on birds, including great tits (e.g. Hõrak et al., 1998; Ots et al., 1998; Norte et al., 2008), greenfinches (*Carduelis chloris*; Hõrak et al., 2002) and Swainson's Hawks (*Buteo swainsoni*; Sarasola et al., 2004). High plasma protein and albumin levels indicate the acquisition of good quality, high protein foods (Jenni-Eiermann and Jenni, 1998; Ardia, 2006). High triglyceride levels indicate fat absorption, while lower plasma triglyceride concentrations are indicative for a fasting state when stored fat needs to be consumed (Jenni-Eiermann and Jenni, 1998). A study of Amat (2007) indicates that elevated cholesterol levels can be indicative for longer periods of fasting. Cholesterol concentrations in yellow-legged gulls (*Larus cachinnans*) have also been related to changes in body mass (Alonso-Alvarez et al., 2002). With this approach of combined antioxidant and nutritional parameter measurements, we could test whether great tits living in metal polluted areas fade because carotenoids are allocated to fight off oxidative stress and/or whether they fade because of differences in diet quality and quantity.

2. Methods

2.1. Study species and study site

Great tits are small songbirds that are widely used in ecological and ecotoxicological research, mainly because they breed and roost in nest boxes (Eens et al., 1999). Great tits have a conspicuous yellow breast. This breast colour is carotenoid-based and caused by deposition of lutein and zeaxanthine (Partali et al., 1987). Great tits derive these carotenoids from their diet, mostly from caterpillars, which are rich in carotenoids.

The study was conducted at four study sites with resident great tit populations in a well-established pollution gradient emanating from a non-ferrous smelter in the South of Antwerp. This smelter is one of Belgium's most extensively metal-polluting point sources for cadmium, lead, arsenic, copper and zinc (VMM, 2006). Pollution originates from uncontrolled emissions from dust of ore piles, which is deposited in close proximity of the smelter. This contaminated dust is only transported over a short distance, rendering very high and local contamination with metals (Dauwe et al., 1999, 2004; Eens et al., 1999; Janssens et al., 2001; Jaspers et al., 2004). Measurements of depositions of lead and cadmium show an exponential increase towards the pollution source (VMM, 1999). In 2005, mean values of lead and cadmium in PM10-dust fraction ranged from respectively 35–381 ng/m^3 and 2–7 ng/m^3 in the area surrounding the smelter and were among the highest values measured in Belgium and surrounding European countries (VMM, 2007). Our study sites are located eastwards at different distances from the smelter, namely UM (0–350 m), F8 (400–600 m), F7 (2500 m) and F4 (8500 m; Fig. 1). These sites have been used for research on great tits since 1999 (e.g. Dauwe et al., 1999, 2004; Jaspers et al., 2004) so there is a well-established population of great tits, occupying 30–50 nest boxes in every study site. Special attention was paid in selecting study sites with a similar habitat type (deciduous park sites). Previous research in these study sites reported negative effects of metal pollution on biochemical markers, immunocompetence, behaviour and reproductive success of great tits (Janssens et al., 2003a,b; Snoeijs et al., 2004; Gorissen et al., 2005; Vanparys et al., 2008).

2.2. Field sampling

At all study sites, nest boxes were checked every 2 days from 22nd March 2007 onwards to monitor nest-building progress, egg-laying and the start of incubation. In doing so, we could determine and/or estimate the day of hatching and the age of the nestlings. Adults were captured when nestlings were 7 days old. Nestlings were sampled when 15 days old. Captured adult birds were sexed and aged (first-year versus older birds; Svensson, 1984) and marked with individually numbered metal rings. In total, 76 adult and 328 nestling great tits from 45 different nest boxes were sampled. All birds were weighed with a precision of 0.1 g, using a digital balance and the tarsus length was measured to the nearest 0.1 mm with a digital sliding calliper. Wing length was measured to the nearest 1 mm. Condition was defined as the residual from the linear regression of body mass on tarsus length in order to avoid the effect of body size on body mass (Ots et al., 1998). We collected a blood sample (150–300 μL) from the brachial vein. Blood samples were immediately stored at 4 °C and, within 12 h, plasma and erythrocytes were separated by 10 min centrifugation at 800 *g*. We could not determine colouration on live birds, so we collected approximately 20 yellow breast feathers, from the mid-ventral region from each bird. Breast feathers were stored in paper envelopes protected from sunlight at room temperature until colour measurement (maximum 4 weeks after sampling).

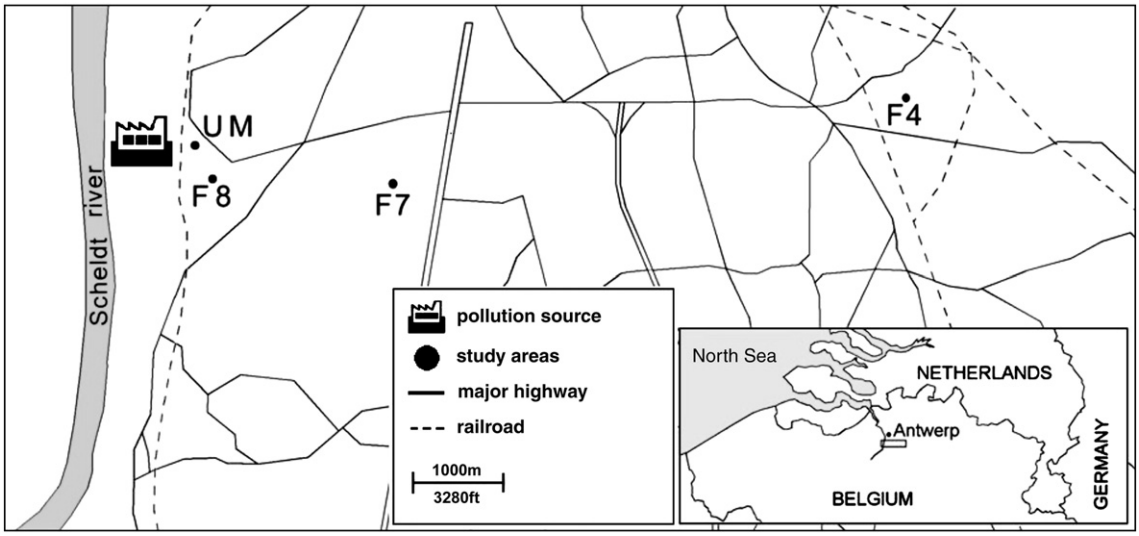

Fig. 1. The location of the four study sites (UM, F8, F7 and F4) along a heavy metal pollution gradient emanating from a large non-ferrous smelter in the south of Antwerp.

2.3. Colour determination

In the laboratory, reflectance spectra (300–700 nm) of the collected feathers were measured with an USB4000 spectrometer (Ocean Optics, Duiven, The Netherlands), using an Ocean Optics DH-2000 BAL deuterium/halogen lamp. All samples were measured in reference to a white reflection standard (WS-1, Diffuse Reflectance Standard, Ocean Optics, Duiven, The Netherlands) and a dark spectrum to eliminate noise. Collected breast feathers were measured as described in Quesada and Senar (2006). Fifteen feathers were placed on a dark velvet surface in five layers of three feathers, trying to imitate the plumage surface of the bird (Quesada and Senar, 2006; Dauwe and Eens, 2008). All feather samples were measured three times by the same person and blind with respect to study site. The mean of these measurements was used for further analysis.

We calculated brightness ($\sum R_{300-700}/n$), hue (the slope between 490 nm and 520 nm) and carotenoid chroma ($R_{700}-R_{450}/R_{700}$) from the spectral data (Andersson and Prager, 2006) for all individuals. Breast feathers of great tits are yellow because of lutein and zeaxanthine (Partali et al., 1987), which have their reflectance spectrum at 450 nm. The higher the carotenoid chroma, the higher the concentration of carotenoids in the feathers (Saks et al., 2003).

2.4. Physiological condition parameters

All plasma samples were analysed with a Cobas MIRA S Plus Chemistry analyser (Horiba ABX, Montpellier, France). Cholesterol, triglyceride, total protein, uric acid and albumin concentrations were measured using standard commercial kits (Horiba ABX). Repeatability (within-run precision) and reproducibility (run-to-run precision) of the measurements are certified by including a calibrator and a control (provided by Horiba ABX) with known concentrations each run and was in all cases between 95 and 105% of the certified value. As the amount of plasma varied, not all parameters could be determined in all individuals. Therefore uric acid was only measured in plasma of nestlings.

As a measure for oxidative stress, we used the Trolox-equivalent antioxidant capacity measure of the plasma (TEAC, Cohen et al., 2007; Hõrak et al., 2007). TEAC was measured as described in Erel (2004) and the method was adapted for the Cobas MIRA S Plus Chemistry analyser. In this assay, ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) is incubated with hydrogen peroxide in an acetate buffer, oxidizing the ABTS to $ABTS^+$ (10 mmol/mL ABTS, pH 3.6). This pro-oxidizing solution has a characteristic stable green colour at low pH. 5 µL of plasma was then diluted with 200 µL of Na-acetate buffer (pH 5.8). 20 µL of the ABTS solution was then added. Because of the higher pH of the solution, the $ABTS^+$ molecules are reduced to ABTS, which results in a decolourization of the solution. The antioxidants in the plasma speed up the reduction of $ABTS^+$ to ABTS, so that the decolourization can be linked to the concentrations of antioxidants in the plasma, or the TEAC. We measured the difference in absorbance at 660 nm (ΔA) between the beginning and end of the 30 min reaction. These ΔA values were then transformed by comparing the ΔA values of the plasma samples with those from a Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a vitamin E analogue) calibration curve. The results are quantified as mM Trolox equivalents per liter.

Because of the limited amount of plasma and the high number of parameters we wanted to measure, we did not have enough plasma to also measure plasma carotenoid concentrations. Although this is also an important parameter, we decided that in the scope of this research the physiological condition parameters together with the carotenoid chroma of the feathers would be sufficient.

2.5. Statistical analyses

Data were analysed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Normality of the data was tested with a Shapiro–Wilk test ($p<0.05$). All tests were general linear mixed models and linear regressions. Data for adult and nestling birds were analysed separately. For all analyses (both for colour and physiological condition parameters), we started with the full model and used backward elimination at $p>0.05$ level. Full models for adults included study site, sex, age, date of capture, body mass, body condition, tarsus length and brood size. For nestlings, we included study site, date of capture, body mass, body condition, tarsus length, brood (i.e. the nestbox where they were reared) and brood size in the full model. Significant differences were tested post-hoc with a Tukey HST test. Sample sizes for different analyses are not always the same, because not all parameters could be measured for all samples due to limitations in the amount of plasma. Outliers in the data (data points that are at least three standard deviations larger than the mean) were removed prior to analyses.

3. Results

For adults as well as for nestlings, brood size, date of capture, body mass, body condition and tarsus length never had a significant ($p<0.05$) effect so we excluded them from all further analyses. After backward elimination, all models for adults only included study site as a

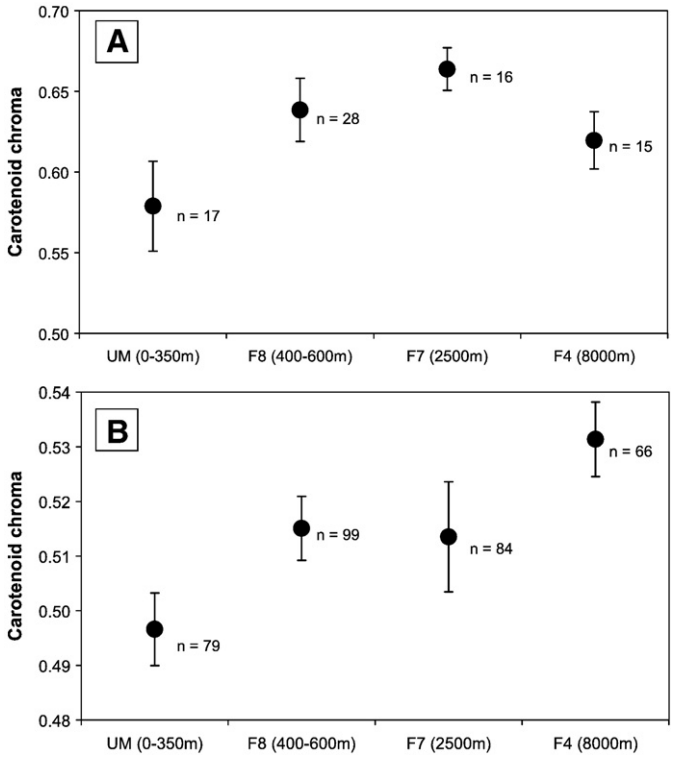

Fig. 2. Carotenoid chroma for adult (A, $n=76$) and nestling (B, $n=328$) great tits from study sites located at different distances from a metal pollution source. Higher carotenoid chroma indicates higher carotenoid concentration in the feathers. Means ± SE are presented.

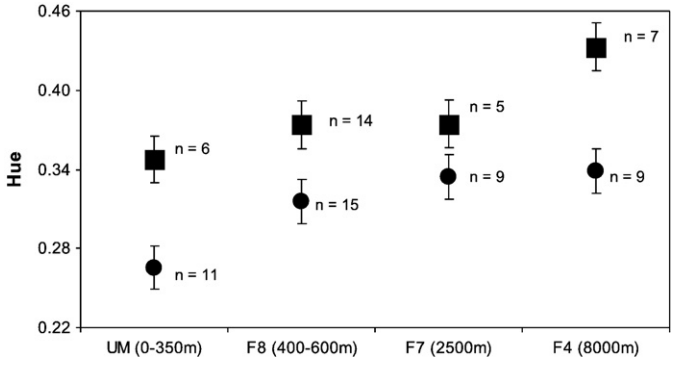

Fig. 3. Hue of male (squares, $n=32$) and female (circles, $n=44$) adult great tits from study sites at different distances from a metal pollution source. Means ± SE are presented.

fixed factor, together with age and sex. Models for nestlings resulted, after backward elimination, in a model with only study site as a fixed factor and brood as a random factor, nested within study site.

3.1. Adults

Male great tits were larger (tarsus length: $F_{1,55}=18.5$, $p<0.001$, $n=57$; wing length: $F_{1,55}=42.25$, $p<0.001$, $n=57$), but not heavier ($F_{1,55}=2.7$, $p=0.1$, $n=57$) than females. There were no significant differences between age groups or among sites for tarsus, wing length and body mass ($F<1.93$, $p>0.1$, $n=57$ in all cases). There were no differences in condition among sites or among age groups and sexes ($F<0.46$, $p>0.7$, $n=57$ in all cases).

Adult carotenoid chroma differed significantly among birds from the four study sites in the pollution gradient ($F_{3,72}=6.3$, $p=0.001$, Fig. 2, Table 1). Post-hoc tests revealed that great tits from the most polluted site UM (0–350 m from the pollution source) had a significantly lower carotenoid chroma than great tits from F7 (2500 m) and F4 (8000 m; $p<0.004$ in both cases). There was no significant difference in carotenoid chroma between males and females ($F=0.03$, $p=0.9$; Table 1) or between age classes ($F=1.6$, $p=0.2$). The hue of adults was significantly lower at the most polluted site UM than at the three other sites ($F_{3,71}=2.8$, $p=0.05$, Table 1), and males had overall higher hue values than females ($F_{1,71}=12.7$, $p<0.001$; Fig. 3, Table 1). Adult males were brighter (22.1 ± 0.4) than females (20.2 ± 0.3; $F_{1,75}=9.9$, $p=0.002$, Table 1), but brightness did not differ significantly among study sites ($F_{3,75}=1.2$, $p=0.3$, Table 1).

There were no significant differences in total protein, albumin, triglyceride and cholesterol concentrations or in TEAC among study sites (Table 2). Concentrations of males did not differ from those from females for the measured physiological condition parameters ($F<4.2$, $p>0.5$ in all cases). One year old adults had significantly lower albumin concentrations (9.4 ± 0.5 g/L) than older adults (12.4 ± 0.3 g/L; $F_{1,61}=13.6$, $p<0.001$). Contrary to expectation, we found no significant relation between carotenoid chroma and TEAC ($F_{1,47}=0.166$, $p=0.7$; Fig. 4). Hue was however significantly negatively related to TEAC, while albumin had a significant positive effect (linear regression with hue as dependent factor: TEAC: $\beta=-0.27$, $F_{1,\ 48}=0.022$, $p=0.05$; sex: $\beta=-0.43$, $F_{1,48}=0.063$, $p=0.002$; albumin: $\beta=0.27$, $F_{1,48}=4.26$, $p=0.04$; Fig. 5). We also found a significant negative effect of TEAC, together with sex, on brightness (linear regression with brightness as dependent factor: TEAC: $\beta=-0.26$, $F_{1,49}=3.9$, $p=0.05$; sex: $\beta=-0.41$, $F_{1,49}=9.9$, $p=0.003$).

3.2. Nestlings

Hatching success and the number of chicks per brood did not differ among study sites ($F<1.9$, $p>0.1$ in both cases). The weight of nestlings however differed significantly among study sites ($F_{3,310}=9.9$, $p<0.001$, $n=314$). Post-hoc tests revealed that nestlings from the most polluted sites UM and F8 had a significantly lower body

Table 1
Results of the ANOVA, means and standard errors of colour parameters for adult great tits in a pollution gradient originating from a metal smelter ($n=76$).

	UM (0–350 m)		F8 (400–600 m)		F7 (2500 m)		F4 (8000 m)		Site			Sex		
	Male ($n=6$)	Female ($n=11$)	Male ($n=14$)	Female ($n=15$)	Male ($n=5$)	Female ($n=9$)	Male ($n=7$)	Female ($n=9$)	df	F	p	df	F	p
Mean brightness	23.28 ± 0.55	19.86 ± 0.63	22.05 ± 0.52	20.97 ± 0.68	19.99 ± 2.06	19.60 ± 1.00	22.86 ± 1.40	19.72 ± 0.89	75	1.2	0.3	75	9.9	*0.002*
Hue	0.35 ± 0.04	0.27 ± 0.03	0.37 ± 0.02	0.32 ± 0.02	0.37 ± 0.06	0.33 ± 0.02	0.43 ± 0.03	0.34 ± 0.02	71	2.8	*0.05*	71	12.7	*<0.001*
	Males and females ($n=17$)		Males and females ($n=29$)		Males and females ($n=14$)		Males and females ($n=16$)							
Carotenoid chroma	0.61 ± 0.03 *A*		0.65 ± 0.01 *AB*		0.69 ± 0.03 *B*		0.70 ± 0.01 *B*		72	6.3	*0.001*	75	0.03	0.9

The letters (A–B) indicate significant differences (Tukey test, $p<0.05$). Letters are not shown if there are no significant differences.

Table 2
Results of the ANOVA, means and standard errors of physiological parameters for adult great tits in a pollution gradient originating from a metal smelter ($n=59$).

	UM (0–350 m)	F8 (400–600 m)	F7 (2500 m)	F4 (8000 m)	Site		
	($n=14$)	($n=20$)	($n=14$)	($n=11$)	df	F	p
Total protein (g/L)	28.5 ± 1.8	24.6 ± 1.1	28.6 ± 1.1	34.6 ± 2.1	3	1.6	0.2
Albumin (g/L)	11.7 ± 0.5	10.6 ± 0.4	12.4 ± 0.4	14.0 ± 0.8	3	2.0	0.1
Triglyceride (mmol/L)	1.7 ± 0.1	1.56 ± 0.09	1.9 ± 0.11	1.91 ± 0.08	3	2.0	0.1
Cholesterol (mmol/L)	4.63 ± 0.2	4.24 ± 0.21	4.94 ± 0.24	5.04 ± 0.22	3	1.9	0.1
TEAC (mmol Trolox equ./L)	3.09 ± 0.25	2.66 ± 0.17	2.76 ± 0.19	2.28 ± 0.42	3	0.1	0.5

mass than nestlings from F7 and F4 ($p<0.001$ in both cases). Tarsus length and body condition on the other hand did not differ significantly among study sites ($F<2.4$, $p>0.1$ in both cases, $n=314$).

Nestling carotenoid chroma differed significantly among study sites ($F_{3,150}=3.4$, $p=0.02$, Fig. 2, Table 3) and among broods ($F_{38,150}=3.9$, $p<0.001$, Table 3). Post-hoc tests revealed that nestlings from the most polluted sites UM and F8 had a significantly lower carotenoid chroma than nestlings from F7 (2500 m) and F4 (8000 m; $p<0.02$ in all cases). The brightness of nestlings differed significantly among broods ($F_{38,150}=2.7$, $p<0.001$, Table 3), but not among study sites ($F_{3,150}=1.3$, $p=0.3$, Table 3). Nestling hue differed among broods ($F_{38,150}=3.2$, $p<0.001$, Table 3) and among study sites ($F_{3,150}=2.6$, $p=0.05$, Table 3), with nestlings from the most polluted site UM having a significantly lower hue than nestlings from the other three sites (post-hoc Tukey test; $p=0.04$).

All physiological condition parameters (TEAC, total protein, albumin, triglyceride and uric acid concentrations), except cholesterol concentrations, differed significantly among broods (Table 4). TEAC, total protein, albumin and uric acid concentrations differed significantly among study sites (Table 4). Triglyceride and cholesterol concentrations of nestlings did not differ significantly among study sites (Table 4). Both uric acid and TEAC were significantly higher at the most polluted sites UM and F8 than at F7 and F4 (post-hoc Tukey test; $p<0.05$; Table 4). Total protein concentrations were significantly higher at the most polluted site UM, compared to the three other sites (post-hoc Tukey test; $p<0.05$; Table 4). Total protein concentrations at F8 were also significantly higher than at F7 and F4, which both did not significantly differ (post-hoc Tukey test; $p<0.05$; Table 4). Contrary to expectations, parameters indicative for nutritional state, were not significantly related to body mass. As in adults, we found no significant relation between carotenoid chroma and TEAC for nestlings ($F_{1,91}=0.23$, $p=0.6$, Fig. 3). We also found no significant relation between the other physiological condition and breast colour parameters in nestlings ($F<3.1$, $p>0.1$ for all parameters).

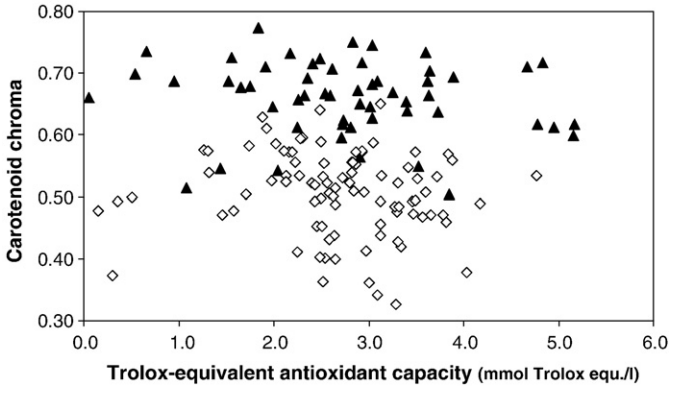

Fig. 4. Relation between carotenoid chroma and TEAC for adult (triangles, $n=59$) and nestling (diamonds, $n=105$) great tits.

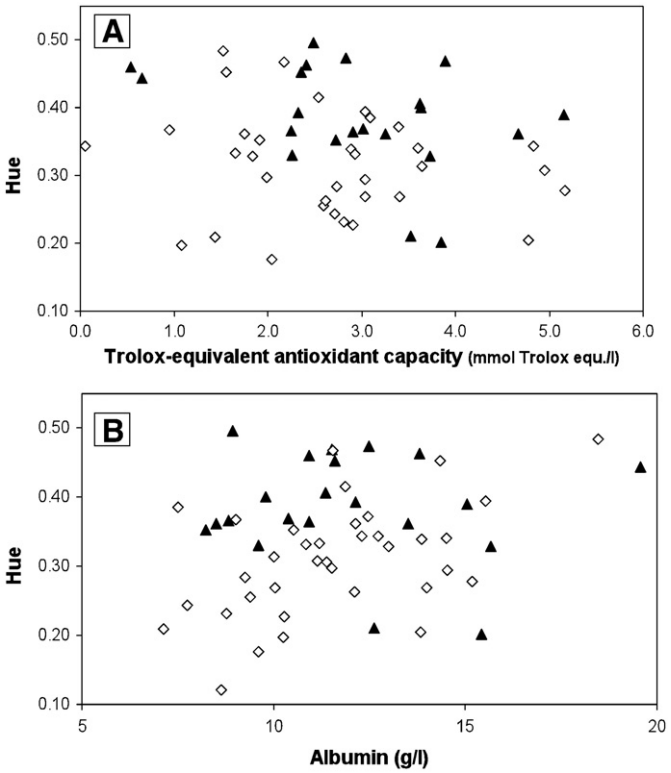

Fig. 5. Relation between hue and TEAC (A) and hue and albumin concentrations (B) for male (triangles, $n=22$) and female (diamonds, $n=37$) adult great tits.

4. Discussion

We tested whether the yellow breast colour of great tits differed among sites with known differences in metal pollution. Both adult and nestling great tits had a significantly less colourful breast at the most polluted sites as compared to the less polluted sites. This finding is in accordance with the results obtained by Eeva et al. (1998) and Isaksson et al. (2007), where great tits inhabiting polluted sites also had paler breast feathers. A study of Dauwe and Eens (2008), conducted in the same pollution gradient as in the present study (but in a different year), confirms this result. As found in Dauwe and Eens (2008), only parameters associated with carotenoid concentrations in feathers (carotenoid chroma and hue) differed significantly among study sites. Brightness, which is moderately related with the carotenoid content of feathers (Saks et al., 2003), did not.

There are several possible, not mutually exclusive, proximate mechanisms for the development of carotenoid colouration (Olson and Owens, 1998). Carotenoid pigmentation could reflect oxidative stress levels or it could reflect dietary carotenoid concentration. Other limiting proximate mechanisms for the development of carotenoid colouration have however also been proposed. Carotenoid colouration could be modulated by genetic constraints, parasites, body condition and the proposed detrimental effects of carotenoids (Olson and Owens, 1998; Bortolotti et al., 2000; Tschirren et al., 2003). Ultimately, this colouration reflects a trade-off between ornamentation and physiological functions. Both Ferns and Hinsley (2008) and Senar et al. (2008) showed that the breast colour of great tits was related to the ability of the signaller to ingest

Table 3
Results of the nested ANOVA, means and standard errors of colour parameters of nestling great tits in a pollution gradient originating from a metal smelter ($n=328$).

	UM (0–350 m)	F8 (400–600 m)	F7 (2500 m)	F4 (8000 m)	Site			Brood (site)		
	($n=79$)	($n=99$)	($n=84$)	($n=66$)	df	F	p	df	F	p
Mean brightness	20.73 ± 0.53	21.47 ± 0.47	21.18 ± 0.56	21.79 ± 0.60	3	1.3	0.3	38	2.7	*<0.001*
Hue	0.25 ± 0.01 *A*	0.28 ± 0.01 *B*	0.27 ± 0.01 *B*	0.28 ± 0.01 *B*	3	2.6	*0.05*	38	3.2	*<0.001*
Carotenoid chroma	0.50 ± 0.01 *A*	0.52 ± 0.01 *A*	0.55 ± 0.01 *B*	0.54 ± 0.01 *B*	3	3.4	*0.02*	38	3.9	*<0.001*

The letters (A–B) indicate significant differences (Tukey test, $p<0.05$). Letters are not shown if there are no significant differences.

carotenoids, its body condition, the local timing of breeding and moulting and its likely parental quality. The genetic component in carotenoid colouration has both been confirmed (Tschirren et al., 2003; Fitze et al., 2003) and denied (Hõrak et al., 2000; Senar et al., 2002). We found very strong among brood variation in nestlings, not only for colour, but for all measured variables (except cholesterol), suggesting that there might be a considerable genetic and/or parental investment factor involved. With our non-experimental data, we however cannot distinguish between environmental and genetic factors. Previous research confirms the relation between body condition and carotenoid colouration in nestlings (Peters et al., 2007; Ewen et al., 2008) and adults (Hegyi et al., 2007), but this was not the case in our study. It is possible that limitations due to body condition were lifted because of the overall good breeding performance in every study site that year.

Metal pollution is known to promote the production of free radicals and to induce oxidative stress (Ercal et al., 2001). Therefore, great tits in the more polluted sites could encounter an allocation problem, because they need to allocate more carotenoids to the antioxidant function. The antioxidant function of carotenoids *in vitro* is confirmed by many studies, but their importance in the antioxidant system and their interaction with other antioxidants *in vivo* is still not clear (Krinsky, 2001). Recently, Costantini and Møller (2008) showed in a meta-analysis that carotenoids explain less than 0.002% of the antioxidant capacity of plasma in birds. Nevertheless, the hypothesis that there is a relation between a carotenoid-dependent signal and oxidative stress levels has gained much support (e.g. von Schantz et al., 1999; Alonso-Alvarez et al., 2004). As a measure of oxidative stress, we measured TEAC of plasma. TEAC reflects the activity and concentration of the complex antioxidant system in the plasma. This system consists of dietary antioxidants (e.g. carotenoids, vitamin C and E) and endogenously produced non-enzymatic antioxidants (e.g. albumin, uric acid, glutathione). Increased generation of free radicals can deplete one or more antioxidants from this system (Halliwell and Gutteridge, 2007). Therefore, we expected that a lower TEAC signals increased free radical production and, possibly, oxidative stress (Halliwell and Gutteridge, 2007). Hence, we expected a positive correlation between TEAC and carotenoid pigmentation. However, we found no significant relationship between the carotenoid chroma and the TEAC, neither for adults, nor for nestlings. This is in agreement with recent findings by Isaksson et al. (2007), where there also was no relation between TEAC and carotenoid chroma for nestling or adult great tits caught in study sites with documented differences in air pollution levels. A potential problem with interpreting these results for adults is that measurements of TEAC were obtained in spring, while carotenoid deposition in feathers occurred the previous autumn, during moult. It is possible that there was a relation between pigmentation and TEAC during the moult in autumn. For nestlings, this problem does not occur because feather growth and measurements of TEAC occurred in the same timeframe. Taking into account that there was also no relation between TEAC and carotenoid chroma for nestlings, it is reasonable to suggest that there is indeed no relation between oxidative stress and carotenoid pigmentation.

For adults, the TEAC levels did not differ among study sites. A reason could be that the metal pollution at our most polluted site does not cause a higher free radical production, which does not seem likely considering the high concentration of toxic metals in the environment and food (Janssens et al., 2001; Dauwe et al., 2004) and because of the earlier reported negative effects of this metal pollution on biochemical markers, immunocompetence, behaviour and reproductive success of great tits (Janssens et al., 2003a,b; Snoeijs et al., 2004; Vanparys et al., 2008). Differences in breeding densities have also been reported as mechanisms for changing haematological and physiological condition parameters (e.g. Kilgas et al., 2006, 2007), but in our study the breeding density is similar for all study sites. Another plausible explanation is that the enzymatic antioxidant system would react more strongly on environmental stress than the non-enzymatic system. In a study of Hoffman et al. (1998), concentrations of enzymatic antioxidants in diving ducks [greater scaup (*Aythya marila*), surf scoters (*Melanitta perspicillata*) and ruddy ducks (*Oxyura jamaicensis*)] increased when the ducks were exposed to high metal concentrations.

Nestling TEAC differed significantly among study sites, with higher TEAC levels in more polluted sites. This is contrary to what we expected. We have to stress that assays of TEAC are useful in getting a global picture of relative antioxidant activities, but these results should be interpreted in the light of the chemistry of the assay (Halliwell and Gutteridge, 2007). TEAC measurements reflect the antioxidative potential of all molecules with antioxidative properties in the plasma. Results may therefore be difficult to interpret when changes occur in various antioxidants, possibly in a different direction. For example, urate levels increase in many human diseases because of alterations in purine metabolism, which in some studies have resulted in rises in plasma TEAC in sick people (Halliwell and Gutteridge,

Table 4
Results of the nested ANOVA, means and standard errors of physiological parameters of nestling great tits in a pollution gradient originating from a metal smelter ($n=213$).

	UM (0–350 m)		F8 (400–600 m)		F7 (2500 m)		F4 (8000 m)		Site			Brood (site)		
	($n=60$)		($n=61$)		($n=55$)		($n=37$)		df	F	p	df	F	p
Total protein (g/L)	40.2 ± 3.4	*A*	30.2 ± 0.9	*B*	33.9 ± 1.4	*C*	28.5 ± 4.1	*C*	3	8	*<0.001*	39	3.1	*<0.001*
Albumin (g/L)	13.3 ± 1.2	*A*	11.0 ± 0.2	*B*	10.6 ± 0.9	*BC*	9.2 ± 0.8	*C*	3	4.4	*0.005*	40	2.2	*<0.001*
Triglyceride (mmol/L)	4.35±0.23		4.41 ± 0.17		4.79 ± 0.44		4.14 ± 0.39		3	0.52	0.7	40	4.2	*<0.001*
Uric acid (μmol/L)	1040 ± 78	*A*	1100 ± 57	*A*	802 ± 121	*B*	721 ± 53	*B*	3	29.4	*<0.001*	27	10.2	*<0.001*
Cholesterol (mmol/L)	3.94 ± 0.16		4.27 ± 0.11		4.44 ± 0.41		3.98 ± 0.33		3	1	0.4	40	1.2	0.3
TEAC (mmol Trolox equ./l)	2.82 ± 0.1	*A*	2.97 ± 0.07	*A*	2.3 ± 0.21	*B*	2.08 ± 0.22	*B*	3	7.8	*<0.001*	39	3.1	*<0.001*

The letters (A–C) indicate significant differences (Tukey test, $p<0.05$). Letters are not shown if there are no significant differences.

2007). In our study, the total albumin and uric acid concentrations in nestlings were higher at the more polluted sites. Both albumin and uric acid are plasma antioxidants (Cohen et al., 2007) and therefore could explain the higher TEAC in more polluted sites. Cohen and McGraw (2009) already showed that TEAC covaries strongly with uric acid levels. Then the question remains why uric acid concentrations are higher in metal polluted sites? Giovannini et al. (2006) have shown that with renal dysfunction, one of the potential consequences of metal pollution, there is an increase in uric acid concentrations in the plasma, thus increasing TEAC. Cohen et al. (2008) also showed that high uric acid concentrations may reflect recent stress, poor condition, or a compensatory response. Therefore Costantini and Verhulst (2009), emphasized in a recent paper that information in antioxidant capacity by itself is not sufficient to make inferences about oxidative stress. Low antioxidant capacity does not necessarily indicate high oxidative stress. In our study, we did not measure a marker of oxidative damage, which makes the interpretation of the TEAC data more intricate. To make inferences about oxidative stress, further research should combine markers of antioxidant capacity with markers of oxidative damage.

We also tested whether there were metal-induced changes in the diet of great tits. Adult and nestling total protein, triglyceride and cholesterol concentrations were comparable to previous reported results in great tits (Hõrak et al., 1998; Ots et al., 1998; Hoff et al., 2005). However, albumin concentrations were lower than those previously reported (9.2–14.0 g/L in our study compared to e.g. 20.5–23.5 g/L in Hõrak et al., 1998). Hõrak et al., 1998 state that they had difficulties in separating the prealbumin fraction from the albumin, and that they therefore summed concentrations for both and termed them albumin concentration. Therefore, it is likely that the albumin concentrations reported by Hõrak et al. (1998) are higher than the actual albumin concentrations. The Horiba ABX albumin assay that we used does not have this problem. As a result, it is possible that the different assays for measuring albumin explain the great difference between the albumin concentrations reported in our study and that of Hõrak et al. (1998). To our knowledge, this study is the first to measure uric acid concentrations in great tits, so we could not compare our results with other studies. Nestling albumin levels were significantly higher in more polluted sites. Higher albumin, total protein and uric acid levels can also be indicative for higher protein levels in the diet (Jenni-Eiermann and Jenni, 1998). Our physiological measurements suggest that nestlings at the polluted site have a diet that is richer in proteins. Eeva et al. (1998) found that there are fewer caterpillars in metal polluted sites. Caterpillars are especially rich in carotenoids ($\pm$0.520 g/kg) compared to other invertebrates that make up the diet of nestling great tits (spiders: $\pm$0.280 g/kg, beetles: 0.310 g/kg; Olson, 2006). Spiders (552 $\pm$ 24 g/kg dry mass) and beetles (505 $\pm$ 3 g/kg dry mass) are however richer in proteins than caterpillars (474 $\pm$ 39 g/kg dry mass; Ramsay and Houston, 2003). Nestlings at polluted sites could have a diet that contains more spiders and beetles (and thus less carotenoids and more proteins). This hypothesis is supported by a study of Eeva et al. (2005) where great tits took more beetles and variable “flying insects” and less caterpillars and moths in the polluted area as compared to the unpolluted one. This alteration of diet composition could explain the lower carotenoid chroma and higher albumin and uric acid concentrations of nestlings at polluted sites. Because neither nestling cholesterol (indicative for periods of fasting and for changes in body mass; Alonso-Alvarez et al., 2002; Amat et al., 2007), nor triglyceride concentrations (indicative for fasting state; Jenni-Eiermann and Jenni, 1998) differed among study sites, our results seem to suggest that nestling diet differs in composition but not in quantity among our study sites. To further test this hypothesis, detailed studies of nestling provisioning and food availability should be conducted in the future. Metal pollution might also affect carotenoid synthesis in plants (Rai et al., 2005), which will affect concentrations in the entire food chain. Although scarcely investigated, Isaksson and Andersson (2007) reported significantly lower carotenoid concentrations in caterpillars in more polluted urban sites.

We did not find any differences in total protein, albumin, triglyceride and cholesterol concentrations in adults among our study sites. This suggests that, in contrast to nestlings, adult diet is the same in all study sites in the period under study. When comparing adult and nestling triglyceride, total protein and albumin concentrations, it is noticeable that adult concentrations are always lower. This shows that nestlings are fattening (Jenni-Eiermann and Jenni, 1998) in comparison to adults. Brood rearing is a stressful time for great tits. Due to the high activity and increased metabolism of adults, parents loose weight during this time (Gosler, 1993; Gebhardt-Henrich et al., 1998; Rheinwald, 1981). Under such physiological demanding conditions, existing differences in diet quality might not be reflected easily in plasmatic indices. It is obviously also possible that there are simply no differences in diet quality among sites. Furthermore, due to small sample sizes, we should be cautious in interpreting these results.

Despite the fact that we did not find a relation between TEAC and carotenoid chroma for nestlings or adults, we did find a significant negative relation between TEAC and brightness and hue for adults. This relation was not significant for nestlings. It is not clear at present why there is a negative relation between TEAC and hue. It might be possible that this relation is merely an effect of the opposite effect of metal pollution on both TEAC and hue. Intriginly however, Isaksson et al. (2007) also reported a negative relation between hue and TEAC. In our data, hue was also positively related to albumin concentrations. Senar et al. (2003) found a positive relation between hue and the tail feather growth rate in great tits. Feather growth rate is dependent of diet quality (Grubb, 1995). Because in our study adult tits with higher hues also had higher albumin concentrations, our data suggest that hue is also positively influenced by diet quality. Although both Isaksson et al. (2007) and Senar et al. (2003) used different methods to determine hue, it is interesting to find similar results warranting further research into the information content of hue.

Our study confirms previous studies reporting changes of plumage colour of great tits in metal polluted areas. The mechanism that induces this change of the carotenoid colouration is however not clear. The importance of carotenoid signalling is supported by two hypotheses that link carotenoid colouration to oxidative stress on the one hand and to diet availability on the other. Our study suggests that there is no clear link between carotenoid colouration and the TEAC of the plasma. The hypothesis that carotenoid colouration is affected by metal-induced differences in diet composition and diet quality of great tits could not be rejected, although our results are not conclusive. Further research should extensively investigate the effect of metal pollution on the carotenoid availability and composition of the diet of great tits.

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