Environmental Research 111 (2011) 362–370

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Metal pollution indirectly increases oxidative stress in great tit (*Parus major*) nestlings ☆, ☆☆

Miia J. Koivula [a,*], Mirella Kanerva [b], Juha-Pekka Salminen [c], Mikko Nikinmaa [b], Tapio Eeva [a]

[a] Department of Biology, Section of Ecology, University of Turku, FI-20014 Turku, Finland
[b] Department of Biology, Division of Genetics and Physiology, University of Turku, FI-20014 Turku, Finland
[c] Laboratory of Organic Chemistry and Chemical Biology, 20014 University of Turku, FI-20014 Turku, Finland

ARTICLE INFO

Article history:
Received 6 August 2010
Received in revised form
21 December 2010
Accepted 6 January 2011
Available online 4 February 2011

Keywords:
Enzyme activities
Great tit
Metals
Oxidative stress
Plumage colour

ABSTRACT

Metals can cause oxidative stress by increasing the formation of reactive oxygen species (ROS), when there are insufficient amount of antioxidants to defend against the growing amount of free radicals. We aimed to find out the most reliable biomarkers to detect pollution-related oxidative stress in wild birds by comparing oxidative stress status in great tit (*Parus major*) nestlings at populations in polluted and unpolluted areas. We also studied with experimental manipulations whether dietary carotenoid levels have any role in great tits' antioxidant defence and whether their carotenoid-based plumage colour was connected to an oxidative stress status. We used antioxidants (GSH, carotenoids) and several antioxidant enzymes (GP, GR, GST, SOD, and CAT) as indicators of the oxidative stress. We found no direct connections between dietary metal exposure and antioxidant or antioxidant enzyme levels. The activity of GP was, however, slightly higher in the polluted environment. This was due to poorer condition and subsequently higher level of oxidative stress in the nestlings in the polluted area. We also found a positive association between GP and an ambient temperature during the nestling period, which may be due to higher metabolic activity of partly poikilothermic nestlings in warm weather. The activity of GST was positively related to the number of nestlings at the sampling time. Fledging success was better in an unpolluted area, where also the nestling body mass was higher. Carotenoid treatment increased the plasma carotenoid concentrations 2.1 fold in carotenoid-supplemented birds, but was not associated with the oxidative stress biomarkers or metal levels. The yellow plumage colour was associated with dietary carotenoid levels in both study areas, but not with the metal exposure or the oxidative stress status. Our results suggest that at the exposure levels found in our study area, the enzyme activities do not indicate metal-related oxidative stress. Instead, GP can be used as an indicator of growth related oxidative stress, which is greater in the polluted area. The activity of this enzyme was, however, not directly related to metal exposure, but more likely to some secondary pollution-related change in the nestling condition.

1. Introduction

Metals are very reactive elements and thus toxic to many organisms when interfering with metabolism and important biochemical reactions (Scheuhammer, 1987; Stohs and Bagchi, 1995; Pinto et al., 2003). Metals can have direct and indirect effects on the wildlife, affecting directly to the egg quality and number, growth rate, nestling condition and morphology of the animals (Hoffman et al., 1985; Nyholm, 1994; Janssens et al., 2003; Bel'skii et al., 2005) as well as an alteration in biochemical processes, such as changes in enzyme activities and free radical levels (Pinto et al., 2003). On the other hand, metal pollution can indirectly change the habitat, community structure and ecological relationship between species (reviewed by Heliövaara and Väisänen, 1993; Kiikkilä, 2003). For example, by changing food chains metal pollution can affect the food quality and quantity and thus negatively affect the survival of animals (Eeva et al., 1997; Eeva and Lehikoinen, 2004). Recent findings suggest that the metal pollution may also increase oxidative stress levels in wild birds (Berglund et al., 2007; Geens et al., 2009; Kamiński et al., 2009). However, it is still unclear whether this is caused by the direct pro-oxidant effect of metals or by some secondary change, e.g. in diet quality.

☆ *Financial support*: the study was financed by the Academy of Finland (T.E. project 8119367) and metal analyses by Boliden Company, Harjavalta, Finland.
☆☆ *Ethic approval*: the study was conducted in full compliance with Finnish laws and regulation, including the licences of the Animal Care & Use Committee of Turku University and Southwest Finland Regional Environment Centre for our studies.
* Corresponding author. Fax: +358 2 333 6550.
E-mail addresses: miikoi@utu.fi (M.J. Koivula), mmkane@utu.fi (M. Kanerva), j-p.salminen@utu.fi (J.-P. Salminen), miknik@utu.fi (M. Nikinmaa), teeva@utu.fi (T. Eeva).

0013-9351/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.envres.2011.01.005

The oxidative stress can be defined as a state, where an antioxidant defence is disturbed by an increased radical formation causing the oxidative damage to biomolecules (Halliwell and Gutteridge, 2007). A variety of oxidative damages are found to be related to the production of reactive oxygen species (ROS) by metals, and therefore an antioxidant defence has an important role in the protection of organisms against metal-induced oxidative stress. ROS themselves are very reactive oxygen-containing molecules produced in oxidation-reduction reactions (Dowling and Simmons, 2009).

Carotenoids, on the other hand, are a group of hydrophobic antioxidants and pigments originated from plants (Halliwell and Gutteridge, 2007). Birds are not able to synthesise carotenoids by themselves, but need to get them from the diet (Monaghan et al., 2009). Therefore, the quality of the food is essential for the carotenoid intake and an effective antioxidant defence of the birds (Palozza, 1998; Pérez-Rodríquez, 2009). Carotenoids are essential for the formation of the yellow plumage colour in great tits (Partali et al., 1985). The yellow colour has been shown to vary between polluted/urban and unpolluted areas, great tit nestlings being paler in polluted environments (Eeva et al., 1998; Hõrak et al., 2001; Isaksson et al., 2005). This may be caused by the amount of carotenoid-rich caterpillars in their diet, which in some studies has shown to be lower in polluted environments, indicating possible indirect effects of the pollution exposure (Perrins, 1991; Van Noordwijk et al., 1995; Eeva et al., 1997, 1998). Carotenoids can also be important in an antioxidant defence against the oxidative stress and thus the level of carotenoids could reflect the ability of individuals to resist oxidative stress (Lozano, 1994; von Schantz et al., 1999; Alonso-Alvarez et al., 2004). However, some recent studies have argued that not all carotenoids are effective antioxidants, but may have a more important role in other functions as sexual signalling and immune defence (Chew and Park, 2004; Isaksson et al., 2005; Costantini et al., 2006; Tummeleht et al., 2006; Hõrak et al., 2007; Costantini and Møller, 2008; Eeva et al., 2008; Isaksson and Andersson, 2008).

So far, the relation of the oxidative stress to metals has rarely been studied in free-living birds (Koivula and Eeva, 2010). The most commonly used biomarkers for the oxidative stress are antioxidants and antioxidant enzymes. Glutathione (GSH) is one of the most important antioxidant, because of its straight participation in binding with reactive oxygen species (Swiergosz-Kowalewska et al., 2006). Glutathione metabolism has a special role in metal induced oxidative stress because of the functional sulfhydryl group of GSH, which serves as a binding site for many metals (Andrews, 2000; Pinto et al., 2003). Antioxidant enzymes instead, act as catalysts in many important reactions related to an antioxidant defence. The most important ones are glutathione peroxidase (GP), glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD). The main function of antioxidant enzymes is catalysing the breakdown of free radicals and the levels of enzyme activities indicate the degree of oxidative stress (Ercal et al., 2001; Pinto et al., 2003). Because of interspecific differences in the use of antioxidants against ROS, it would be worth to use both enzymatic (e.g. GP, GR, CAT, and SOD) and non-enzymatic (e.g. GSH, carotenoids) antioxidants, when measuring oxidative stress and antioxidant capacity (Costantini and Verhulst, 2009).

In this study, we aimed to find out whether the metal pollution increases oxidative stress levels in nestlings of an insectivorous passerine species, the great tit. Our focus was to find the most reliable biomarkers for great tits to detect the oxidative stress and to study whether birds show an increased oxidative stress in polluted areas as compared to unpolluted ones. The level of reduced glutathione (GSH) gives the overall redox status of the cells (Halliwell and Gutteridge, 2007). Any reduction in its level suggests that the oxidative stress occurs. Such the oxidative stress may result in the reduction of carotenoid levels, as they will be used up to reduce glutathione. The redox-relevant enzymes (GP, GR, GST, SOD and CAT) will also show changes in activity if the animal suffers from the oxidative stress. We also studied experimentally whether dietary carotenoid supplementation has an effect on the oxidative stress by improving the antioxidant defence against ROS production caused by the metal pollution. In addition, to see whether carotenoid-based plumage colouration of the yellow breast feathers of great tits is related to the plasma carotenoid concentration and/or oxidative stress status, we measured the variation in the plumage colour among nestlings. So far there are just a few studies about the relation of plumage colour and oxidative stress by using enzyme activities as biomarkers of oxidative stress levels. In earlier studies, glutathione levels (measured as a ratio of oxidised and reduced glutathione) have shown to indicate the relation of oxidative stress and paler yellow plumage colour in urban areas (Isaksson et al., 2005). However, total antioxidant capacity did not show a clear relation to plumage colouration in great tits (Geens et al., 2009). Besides the effects on the plumage colouration, we wanted to explore the effect of the oxidative stress on the nestling growth, survival and fledging success. Since an ambient temperature affects the metabolic rates of partly poikilothermic nestlings, we further wanted to find out whether the nestling time temperatures are related to the oxidative stress levels.

We hypothesise that (1) the level of oxidative stress is higher in a metal polluted environment due to higher amount of dietary pro-oxidants. (2) Plasma carotenoid concentrations are lower in polluted sites as compared to unpolluted sites, decreasing also the intensity of yellow plumage colour in polluted area. (3) The intensity of the plumage colour in the polluted area is inversely related to an experienced oxidative stress, (4) as well as poor carotenoid availability in their diet. (5) Carotenoid supplementation improves an antioxidant defence and increases the plumage colour intensity in both study areas. (6) Fledging success increases with the nestling condition, being higher in an unpolluted area than in the polluted area.

2. Materials and methods

2.1. Study area

The field studies were conducted in 2004 near a metal smelter in the town of Harjavalta (61°20′ N, 22°10′ E) in the southwest of Finland. The main pollutants in the surrounding area of the smelter are metals such as Cu, Ni, As, Zn and Pb as well as sulphuric oxides from the factories nearby (Kiikkilä, 2003). In the earlier studies of Eeva et al. (1998, 2000) birds are shown to suffer from metal pollution by, e.g., showing decreased fledging success in this area. We had twelve study sites established along the pollution gradient, six sites in the polluted area (<2 km from the smelter) and six sites in the unpolluted area (>5 km from the smelter), where the concentrations of metals are approaching the background levels (Eeva et al., 2008). The habitat type in our study areas was similar barren pine (*Pinus sylvestris*) dominated forest in both polluted and unpolluted areas, to avoid habitat-related variation between the areas.

2.2. Study species

The great tit is an insectivorous resident bird, well suited for the studies of local pollution (Burger, 1993; Eeva, 1996; Janssens et al., 2002). In many studies great tit has been shown to suffer from metal pollution by e.g. having lower breeding success and food depression in the contaminated areas (Eeva et al., 2000; Dauwe et al., 2004; Geens et al., 2009). Also because of the great tits' carotenoid based colouration and omnivorous feeding habits, it is an ideal study object for studies related to carotenoid availability and antioxidant defence. Only nestlings of great tits were used as study objects in this study. The nest boxes were visited regularly from the beginning of the breeding season until the fledgling phase to collect data on the breeding success (hatchling and fledgling numbers). The study was performed under the licences of the Animal Care & Use Committee of the Turku University and Regional Environment Centre for our studies.

2.3. Sample collection and analyses

2.3.1. Temperature data

Temperature data were collected by Finnish Meteorological Institute, using the station within our study area (the meteorological station of Peipohja, Kokemäki, 61°16′ N, 22°15′ E). We used the mean temperature of the preceding nine days (starting from the hatching date) before the blood sampling as a possible confounding factor in the analyses. The nestlings can be considered as partly poikilothermic in their early nestling phase, which might also affect the level of oxidative stress via the metabolic rate or the stress related to cold tolerance.

2.3.2. Carotenoid supplementation

We determined the hatching day by visiting the nest boxes daily starting from two days before the estimated hatching date. When the nestlings were three days old, they were weighed and each brood was divided into two comparable groups for the carotenoid treatment according to their body mass. The groups were randomly chosen for the treatment and control groups. The total number of broods in our experiment was 60 (26 polluted and 34 unpolluted nest sites). The treatment group was treated with water-dispersed carotenoid beadlets (Lutein 5% CWS, Roche, Basel, Switzerland), containing 5% lutein and 0.25% zeaxanthin, which were diluted in distilled water to get a lutein concentration of 5 mg/ml (an oral volume of 0.1 ml/nestling). The control group was treated by giving the same amount of distilled water (a more detailed description of the treatment is given in Eeva et al. (2008)). The treatment increased the plasma lutein level of nestlings' 2.1 × compared to the natural ones, but the levels were still within the range of natural variation (Eeva et al., 2008).

2.3.3. Nestling condition and blood samples

For an individual identification, all nestlings were ringed with aluminium rings (at day 6). The weight and wing length were measured from eight days old nestlings. The body mass and subsequent survival are known to correlate in many birds (e.g. great tits; Perrins, 1965), so the body mass and fledging success (probability of a hatchling to fledge) can be used as a stress marker for the carotenoid treatment and the metal pollution. Growing young birds are thought to be the most sensitive to the detrimental effects of pollutants (Scheuhammer, 1987) and the fledging success has been shown to decrease toward the pollution source in the earlier studies with great tit nestlings (Eeva et al., 2003; Janssens et al., 2003). Blood samples were taken from two randomly selected (excluding exceptionally small ones) nestlings per brood, one from the treatment group and one from the control group, when the nestlings were nine days old. We took the blood sample from the brachial vein, using 75 μl heparinised capillary tubes and centrifuged them immediately for 5 min at 4000 r/min to separate the plasma and red blood cells from each other. Plasma was separated and preserved on ice and kept protected from light during transportation, and stored at −22 °C until the carotenoid analyses. Red blood cells were stored in liquid nitrogen and later kept at −80 °C, until the enzyme activity analyses.

2.3.4. Carotenoid analyses

The plasma carotenoid analyses were determined with high performance liquid chromatography (HPLC). A known volume of plasma (10–35 μl) was extracted 3 × with 100% acetone. The solvent was then evaporated from the combined extract under vacuum and the residue was dissolved into a small volume of 80% acetone. The carotenoid composition of the extracts (lutein, zeaxanthin, β-carotene) was analysed with HPLC at 450 nm, using a Merck Purospher STAR RP-18 (55 × 2 mm, i.d., 3 μm) column (Darmstadt, Germany). β-carotene was quantified as β-carotene and other carotenes as lutein equivalents. In the total carotenoid concentration, lutein, zeaxanthin and β-carotene (the main carotenoids in birds) were identified.

2.3.5. Determination of plumage colour

Plumage colour of the great tits were measured from two randomly selected nestlings per brood (1 treatment, 1 control), when they were 16 days old. The colour was determined by photographing the nestlings side by side with a digital camera, using a uniform grey cardboard as a background and a yellow reference card (C2, M17, Y86 and K0) to determine the colour in each picture. Altogether 57 broods were photographed (24 from polluted sites and 33 from unpolluted sites). Nestlings were placed in a plastic holder to keep them in the same position when photographing the plumage on the ventral side of their body (a more detailed description of the method is given in Eeva et al. (2008)). As long as the ambient lightning can be controlled, the digital imaging has shown to be a sensitive way to measure the colour variation (Villafuerte and Negro, 1998; Montgomerie, 2006). In this study, we focused on the plumage yellowness, and thus the intensity of lutein, which is the main determinant of the yellow colour in the great tit plumage. The proportion (%) of the yellow component (Y_c), which is the corrected value for the plumage colour (calculated by using the reference colour value), was used as a measure of the plumage yellowness. (See more detailed description in Eeva et al., 2008.)

2.3.6. Metal analyses

Faeces from the seven days old defecating nestlings were collected directly to the plastic Eppendorf tubes from 2 to 4 nestlings per brood. The faecal sacs were then combined within the same brood and dried at 50 °C for 72 h. After that, samples were weighed in a range 0.15–0.20 g. The samples were prepared according to the instructions (see Eeva et al., 2008) and the concentration of the metals (Cu, Ni, As, Cd, Pb and Zn) was determined with an ICP-MS (Elan 6100 DRC, PerkinElmer-Sciex, Boston, USA). The detection limit for most of the metals was around or <1 ng/l (a more detailed description of the method is given in Eeva et al. (2008, 2009)). Since all the faecal concentrations of metals were positively correlated to each others, we calculated principal components from the metal data (Ni, Cu, As, Cd, Pb and Zn). Since the first principal component (PC1) explained 65% of the variation in our data, it was used in the models as an explanatory variable to describe the general level of metal exposure (Fig. 1).

2.3.7. Biomarker analyses

Red blood cells from total 113 individuals (50 from polluted sites, 63 from unpolluted sites) were used to measure redox parameters. Samples were defrosted and analysed, using a microplate reader (Envision, Perkin-Elmer Wallac, Turku, Finland). All enzyme activities were measured in triplicates using 96- (CAT) or 384-well (GP, SOD, GR and GSH) microplates to minimise the sample volume. For this reason, the reduction of reagent volumes was often required as compared to the used method instructions. GP, GR, GST and CAT were measured using Sigma kit instructions (Sigma Chemicals, St. Louis, Missouri, USA). SOD was measured with a Fluka kit (Fluka, Buchs, Germany) according to the kit instructions. Total GSH was measured using an OxisResearch kit (Foster City, California). The protein assay was done according to the Bradford method (Bradford, 1976), using the BioRad protein assay (BioRad, Espoo, Finland) and bovine serum albumin (BSA) (Sigma) as a standard.

2.3.8. Statistical analyses

All correlations between the study variables were analysed using Spearman correlation coefficient. These correlation coefficients were used as a tool when building reasonable models for explaining variation along the pollution gradient. The variation in enzyme activities between the areas (polluted vs. unpolluted) and treatment groups (carotenoid vs. water) was studied with generalised linear mixed models (GLMMs with lognormal error distribution and identity link function; see Table 1) in the SAS statistical software 9.2 (SAS Institute 2002–2008). Explaining factors in the models were: area, treatment, area × treatment, body mass, area × body mass, average temperature prior sampling, PC1 of metals, plumage colour and brood size at the sampling date (at the age of nine days; Table 1). Brood was used as a random factor to take account that two nestlings were sampled from the same brood. The degrees-of-freedom were calculated with Kenward-Roger method. Non-significant terms were dropped from the models one-by-one starting from interactions.

The fledging success was analysed with a GLMM (with binomial distribution and logit link function), using area, treatment, area × treatment, body mass and PC1 of metals as explaining factors in the model. Further, to see if the GP activity is associated with fledging success, we used the GP activity, area and area × GP activity interaction as explaining factors in the model. GLMM (with normal distribution and identity link function) was also used to analyse lutein concentration, using area, treatment, area × treatment, body mass, brood size and PC1 of metals as explaining factors. The time of the day was included into this model, because the level of carotenoids can vary during the day depending on the time they have spent to eat carotenoid rich food (Eeva et al., 2008). Plumage colour (Y_c value) was likewise analysed, using GLMM (with normal distribution and

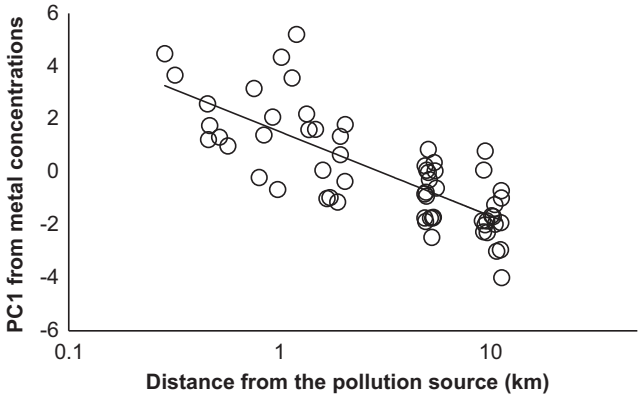

Fig. 1. Metal concentrations in faeces along the pollution gradient. The first principal component (PC1) from the metals (Ni, Cu, As, Cd, Pb and Zn) was used to describe the general level of metal exposure. Note a logarithmic scale of the *X*-axis.

Table 1
Mean enzyme activities (glutathione peroxidase, GP; glutathione reductase, GR; glutathione-S-transferase, GST; catalase, CAT; superoxide dismutase, SOD) and glutathione (GSH) levels in blood of nine days old *Parus major* nestlings for lutein supplemented (lutein) and control (water) groups in polluted and unpolluted areas. Non-significant terms were dropped one-by-one from generalised linear models. The terms left in the final models are shown in bold.

Group		**GP** (nmol/min/mg)		**GR** (nmol/min/mg)		**GST** (nmol/min/mg)		**GSH** (μmol/mg)		**CAT** (μmol/min/mg)		**SOD** (inhibition %)	
		n	Mean (95% CI)	*n*	Mean (95% CI)	*n*	Mean (95% CI)	*n*	Mean (95% CI)	*n*	Mean (95% CI)	*n*	Mean (95% CI)
Polluted	*Lutein*	25	0.77 (0.63–0.91)	25	5.13 (3.94–6.33)	25	3.07 (2.38–3.76)	25	12.33 (7.55–17.10)	25	55.32 (43.24–67.40)	25	26.26 (21.27–31.24)
	Water	25	0.94 (0.68–1.20)	25	6.03 (4.37–7.70)	25	3.85 (2.44–5.26)	25	12.08 (9.26–14.91)	24	58.54 (44.24–72.85)	25	25.95 (21.30–30.61)
Unpolluted	*Lutein*	32	0.73 (0.51–0.96)	32	5.31 (4.25–6.36)	32	3.81 (2.87–4.76)	32	18.95 (7.98–29.93)	32	53.21 (43.92–62.49)	32	24.54 (19.57–29.52)
	Water	31	0.76 (0.58–0.93)	31	5.27 (4.02–6.52)	31	3.92 (2.67–5.17)	31	10.09 (7.79–12.38)	31	49.49 (40.73–58.25)	31	25.96 (21.78–30.13)
Source of variation		**F_{df}[a]**	***p***	**F_{df}[a]**	***p***	**F_{df}[a]**	***p***	**F_{df}[a]**	***p***	**F_{df}[a]**	***p***	**F_{df}[a]**	***p***
Area		**$3.94_{1,\ 57.14}$**	**0.052**	$0.50_{1,\ 48.7}$	0.483	$1.15_{1,\ 50.21}$	0.288	$0.03_{1,\ 49.66}$	0.870	$0.55_{1,\ 47.61}$	0.463	$3.32_{1,\ 53.99}$	0.074
Treatment		**$0.74_{1,\ 77.82}$**	**0.391**	$0.23_{1,\ 83.83}$	0.633	$1.48_{1,\ 86.03}$	0.227	$0.09_{1,\ 86.07}$	0.764	$2.11_{1,\ 83.03}$	0.150	$0.94_{1,\ 55.53}$	0.336
Area × treatment		$0.11_{1,\ 53.86}$	0.743	$0.72_{1,\ 51.63}$	0.401	$0.88_{1,\ 52.3}$	0.353	$0.76_{1,\ 53.43}$	0.388	$2.00_{1,\ 51.45}$	0.164	$0.52_{1,\ 51.59}$	0.473
Body mass (at age 9)		**$0.10_{1,\ 56.95}$**	**0.758**	$0.30_{1,\ 69.78}$	0.585	$1.30_{1,\ 79.64}$	0.257	$1.32_{1,\ 73.56}$	0.254	$0.00_{1,\ 66.61}$	0.999	$1.10_{1,\ 70.47}$	0.298
Area × body mass		**$6.50_{1,\ 58.1}$**	**0.014**	$0.89_{1,\ 69.22}$	0.350	$0.01_{1,\ 77.63}$	0.936	$3.55_{1,\ 76.12}$	0.063	$1.05_{1,\ 74.13}$	0.309	$0.44_{1,\ 66.71}$	0.507
Temperature		**$10.4_{1,\ 46.3}$**	**0.002**	$0.06_{1,\ 47.13}$	0.812	$0.00_{1,\ 48.01}$	0.985	$2.65_{1,\ 52.58}$	1.110	$1.40_{1,\ 54.86}$	0.242	$0.01_{1,\ 46.11}$	0.934
Brood size		**$0.01_{1,\ 50.45}$**	**0.915**	$2.48_{1,\ 60.77}$	0.120	**$3.89_{1,\ 59.96}$**	**0.053**	$1.61_{1,\ 59.35}$	0.210	$0.86_{1,\ 57.12}$	0.357	$0.28_{1,\ 58.68}$	0.600
Faecal heavy metal level (PC1)		**$0.61_{1,\ 47.32}$**	**0.437**	$0.00_{1,\ 47.37}$	0.977	$0.01_{1,\ 50.4}$	0.931	$0.01_{1,\ 50.11}$	0.922	$0.76_{1,\ 49.42}$	0.388	$0.60_{1,\ 56.75}$	0.443
Plumage colour index		**$0.00_{1,\ 61.71}$**	**0.962**	$0.46_{1,\ 103}$	0.498	$3.13_{1,\ 98.67}$	0.080	$0.14_{1,\ 99.9}$	0.710	$2.22_{1,\ 102.8}$	0.139	$0.06_{1,\ 76.13}$	0.803

[a] General linear mixed models (lognormal distribution, identity link function), nest box used as a random factor in the model.

identity link function), where the explaining factors in the model were: area, treatment, area × treatment, body mass, brood size and PC1 of metals. One-way ANOVA was used to test the significance of metal concentrations between the study areas. Carotenoid concentration was log transformed and metal concentrations $\log_{10}$ transformed before the analyses to normalise distributions.

3. Results

Faecal metal levels (PC1) did not significantly explain the variation in any of the studied enzyme activities (GP, GR, GST, GSH, CAT and SOD; Table 1). However, the area and the nestling body mass showed a significant interaction on the GP activity, suggesting higher GP activity in the polluted area, where the nestling body mass is lower (Table 1). In the unpolluted area, heavy nestlings showed lower activities, indicating that the variation in the GP activity among areas is condition dependent (Table 1). The GP activity also increased with ambient nestling time temperature in both the areas (Table 1, Fig. 2). The GST activity was positively associated with the number of nestlings (Table 1, Fig. 3), showing higher values when the number of nestlings were higher, though the result was only marginally significant. Other enzyme activities did not vary significantly between the study areas, nor there were any association to temperature, nestling body mass or plumage colour (Table 1). Some of the enzyme activities were correlated with each other, showing significant correlation when enzymes were functionally closely linked together (e.g. GSH-GP, CAT-SOD, and GSH-GST) in detoxifying free radicals (Table 3).

Fledging success was significantly better in an unpolluted area than in the polluted area (Table 2). Increased metal concentration

Fig. 2. Blood glutathione peroxidase (GP) activity of nine days old *P. major* nestlings in relation to an ambient nestling time temperature.

Fig. 3. Blood glutathione-S-transferase (GST) activity relative to the number of nestlings in the great tit broods.

Table 2
Mean plasma lutein concentrations, plumage colour index and fledging success (probability of hatchlings to fledge) of *Parus major* nestlings for lutein supplemented (lutein) and control (water) groups in polluted and unpolluted areas. Non-significant terms were dropped one-by-one from generalised linear models. The terms left in the final models are shown in bold.

		Lutein (μg/ml)		**Plumage colour (Y_c-value)**		**Fledging success (%)**	
		n	Mean (95% Cl for means)	*n*	Mean (95% Cl for means)	*n*	Mean (95% Cl for means)
Polluted	*Lutein*	26	66.4 (52.7–80.1)	24	81.2 (78.4–84.1)	26	56.5 (46.6–66.4)
	Water	26	28.9 (20.7–37.0)	23	70.3 (65.8–74.8)	26	56.5 (46.6–66.4)
Unpolluted	*Lutein*	34	53.7 (42.8–64.7)	33	83.3 (81.2–85.5)	34	71.1 (62.5–79.7)
	Water	33	27.3 (18.9–35.8)	33	72.8 (69.2–76.5)	33	73.3 (65.6–80.9)
Source of variation		**F_{df}[a]**	***p***	**F_{df}[a]**	***p***	**F_{df}[b]**	***p***
Area		**$6.27_{1,\ 56.83}$**	**0.015**	$0.35_{1,\ 54.72}$	0.559	**$10.4_{1,\ 115}$**	**0.002**
Treatment		**$44.8_{1,\ 58.37}$**	**<0.0001**	**$114.0_{1,\ 55}$**	**<0.0001**	$0.02_{1,114}$	0.879
Area × treatment		$0.00_{1,\ 56.67}$	0.972	$0.07_{1,\ 54.59}$	0.793	$0.00_{1,113}$	0.969
Time of day		**$14.3_{1,\ 56.92}$**	**0.0004**	–	–	–	–
Body mass (at age 9)		$0.03_{1,\ 77.48}$	0.865	$1.21_{1,\ 92.7}$	0.274	**$41.5_{1,\ 115}$**	**<.0001**
Brood size		$0.00_{1,\ 60}$	0.990	**$16.7_{1,\ 57.87}$**	**0.0001**	–	–
Metals (PC1)		$0.07_{1,\ 56.56}$	0.787	$0.05_{1,\ 54.59}$	0.824	**$3.63_{1,115}$**	**0.059**

[a] General linear mixed models (normal distribution, identity link function), nest box used as a random factor.
[b] General linear mixed models (binomial distribution, logit link function)

Table 3
Spearman correlation coefficients (*r*-value, *p*-value and number of individuals) for enzyme activities (glutathione peroxidase, GP; glutathione reductase, GR; glutathione-S-transferase, GST; catalase, CAT; superoxide dismutase, SOD), glutathione (GSH) and plasma lutein concentrations, plumage colour, fledging success (% hatchlings fledging) and nestling body mass. These correlation coefficients were used as a tool when building reasonable models for explaining variation along the pollution gradient and no tablewise corrections for α-levels were applied.

Variables	GP (nmol/min/mg)	GR (nmol/min/mg)	GST (nmol/min/mg)	GSH (μmol/mg)	CAT (μmol/min/mg)	SOD (inhibition %)	Lutein (μg/ml)	Colour (Y_c-value)	Fledging succ. (%)	Body mass
GR (nmol/min/mg)	0.07 0.61 (57)	–	–	–	–	–	–	–	–	–
GST (nmol/min/mg)	0.08 0.56 (57)	0.57 **<0.0001 (57)**	–	–	–	–	–	–	–	–
GSH (μmol/mg)	**0.30** **0.02 (57)**	−0.24 0.07 (57)	**−0.50** **<0.0001 (57)**	–	–	–	–	–	–	–
CAT (μmol/min/mg)	−0.16 0.22 (57)	**−0.48** **0.0002 (57)**	**−0.42** **0.001 (57)**	−0.03 0.85 (57)	–	–	–	–	–	–
SOD (inhibition %)	−0.02 0.87 (57)	**−0.37** **0.005 (57)**	**−0.43** **0.001 (57)**	0.22 0.11 (57)	**0.42** **0.001 (57)**	–	–	–	–	–
Lutein (μg/ml)	0.02 0.90 (57)	−0.11 0.41 (57)	−0.21 0.11 (57)	−0.001 0.99 (57)	0.14 0.30 (57)	0.14 0.30 (57)	–	–	–	–
Colour (Y_c–value)	−0.10 0.45 (55)	−0.07 0.62 (55)	−0.18 0.19 (55)	−0.18 0.18 (55)	0.16 0.26 (55)	−0.18 0.19 (55)	0.13 0.32 (58)	–	–	–
Fledging success (%)	**−0.48** **0.0001 (57)**	0.03 0.85 (57)	0.12 0.36 (57)	−0.04 0.76 (57)	−0.02 0.87 (57)	−0.05 0.74 (57)	−0.003 0.98 (60)	0.04 0.75 (58)	–	–
Body mass (g)	−0.16 0.24 (57)	0.13 0.34 (57)	**0.28** **0.03 (57)**	−0.16 0.24 (57)	−0.10 0.45 (57)	**−0.28** **0.03 (57)**	−0.17 0.20 (60)	0.15 0.25 (58)	**0.59** **<0.0001 (60)**	**–**
Brood size	−0.18 0.19 (57)	0.16 0.23 (57)	**0.25** **0.05 (57)**	−0.23 0.08 (57)	−0.09 0.53 (57)	−0.07 0.63 (57)	−0.07 0.61 (60)	**0.37** **0.004 (58)**	**0.27** **0.04 (60)**	**0.26** **0.05 (60)**

was associated with decreased fledging success, but the effect was only marginally significant (Table 2). Faecal metal concentrations instead differed significantly from each other between the study areas (Table 4). Also body mass was positively associated with fledging success, but carotenoid treatment did not affect the fledging success (Table 2). Based on our result of an increased GP activity along the temperature gradient and differences between the study areas, we chose the GP activity for more detailed examination. The probability of fledging decreased in nests with increased nestling GP activity (GLMM: $F=12.67_{1,\ 53,}$ $p=0.0008$) and the study areas differed significantly from each other (GLMM: $F=12.07_{1,\ 53}$, $p=0.0010$; Fig. 4), indicating stronger association between nestling mortality and the GP activity in the unpolluted area than in the polluted area, where the total nestling mortality is higher. There was no association between any other enzyme activities and the likelihood of a hatchling to fledge in our study areas.

Carotenoid treatment increased the plasma lutein concentration 2.1 fold in carotenoid-supplemented birds compared to the control birds (Table 2) and it was also 1.2 × higher in polluted sites compared to unpolluted sites (Table 2). We did not find any interaction on plasma lutein levels between carotenoid treatment and the study area, indicating that the carotenoid treatment produced the same response in two environments (Table 2). However, the lutein concentrations increased during a day (Table 2). No associations between carotenoid treatment and enzyme activities were found (Table 3).

The yellowness (the proportion of the yellow component Y_c) of nestling plumage had no correlation with PC1 of faecal metal concentrations ($r_S= -0.07$, $p=0.59$, $n=58$). Carotenoid treatment, however, increased the yellowness of the plumage in the lutein supplemented birds compared to controls, but there was no significant difference between the study areas (Table 2). Plumage

Table 4
Mean (± standard error) metal concentrations (μg/g; dry weight) in the faeces of *Parus major* nestlings in polluted ($n=56$ nests) and unpolluted ($n=69$ nests) areas.

Metal	Polluted	Unpolluted	F_{df}[a]	p
Ni	35.1 ± 3.2	7.2 ± 0.5	91.0_1	< 0.0001
Cu	232 ± 20.3	111 ± 6.6	38.0_1	< 0.0001
As	12.9 ± 1.7	1.7 ± 0.1	52.7_1	< 0.0001
Cd	3.8 ± 0.4	1.8 ± 0.1	22.2_1	< 0.0001
Pb	5.1 ± 0.6	2.7 ± 0.3	13.1_1	0.0004
Zn	389 ± 21.3	338 ± 11.7	5.0_1	0.027

[a] One-way ANOVA used to test the significance between areas.

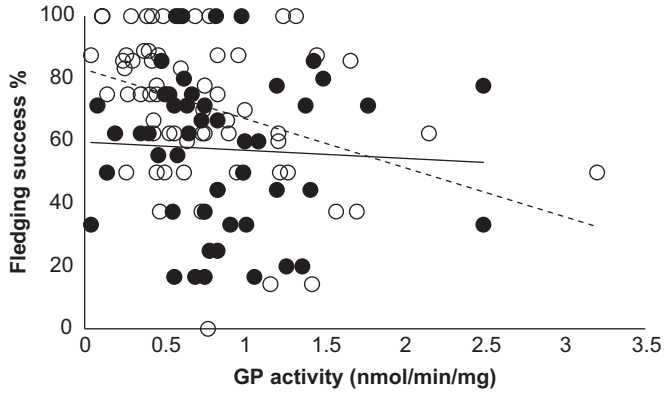

Fig. 4. The variation of fledging success between unpolluted (open circles, dash line) and polluted areas (solid circles, solid line) relative to GP activity.

colour also increased significantly with the brood size (Table 2). None of the enzyme activities showed significant association to the plumage colour (Table 3).

4. Discussion

4.1. Oxidative stress and indirect effects of metals

4.1.1. Metals and enzyme activities

Our results showed that dietary metal concentrations have no effect on the enzyme activities in great tit nestlings along the pollution gradient at the levels occurring in our study area. Also the lack of changes in the total GSH concentration between the study areas indicates low oxidative stress in the nestlings. Most likely the metal concentrations in our study site are not high enough to cause oxidative stress in the great tit nestlings. The comparison of different metal concentrations among studies is rather difficult; not only because of just a few studies done in birds concerning metals and oxidative stress, but also because of the variation between the methods and tissues used to determine the metal concentrations. Different tissues accumulate different amounts of metals, liver or kidney, for example, less than faeces (Dmowski, 1993), so the direct comparison cannot be done between tissues. However, our metal concentrations are relatively low compared to other studies done in birds near the pollution source (Dmowski, 1993; Bel'skii et al., 1995; Janssens et al., 2003; Berglund et al., 2007). To know the exact metal concentrations that cause oxidative stress would need more comparable studies, where oxidative stress biomarkers are included. The faecal material as an indicator of metal pollution (consisting of unabsorbed and excreted absorbed metals) has shown to reflect the metal pollution level well in environment and food items, indicating especially the food chain contamination (Eeva and Lehikoinen, 1996; Dauwe et al., 2000; Dauwe et al., 2004). Faeces metal concentration, however, tells less about tissue concentrations (Berglund, 2010), but the use of the tissues brings the ethical problems because of a need of terminal sampling (Garlick et al., 1987). Our own comparison of metal concentrations in liver and in faeces, however, correlated well with each other (unpublished data). Feathers are also commonly used to measure the metal contamination, but they are not suitable for all metals (e.g. Cu, As and Cd) (Dauwe et al., 2000), whereas blood concentrations of metals reflect mainly just newly ingested metals (Berglund, 2010). Our results are in accordance with the studies of Isaksson et al. (2009), who studied oxidative stress levels in great tits in urban vs. rural habitats. They did not either find any difference in the studied antioxidant enzyme activities (CAT, GST, and GR) between the study areas (Isaksson et al., 2009). In contrast, the studies in another insectivore, pied flycatchers (*Ficedula hypoleuca*), showed an increase in the enzymatic activity of antioxidant enzymes CAT and GR related to high toxic metal concentration close to the pollution source, where the lead concentration has been extremely high (Berglund et al., 2007). Also white stork chicks (*Ciconia ciconia*) have shown to have higher enzyme activities in more polluted environments (Kamiński et al., 2009). In addition to the variation in exposure there is also species specific variation in susceptibility to the oxidative stress as well as in their metabolic rate and requirements of different antioxidants (Pérez-Rodríguez, 2009). This makes it still more complicated to directly compare results of the studies of different species in pollution-related oxidative stress. Our results suggest that metals did not directly increase oxidative stress in our study area, though environmental pollution can indirectly, via changes in the diet composition, decrease the survival of nestlings in the polluted area (see Eeva et al. (2005)).

Antioxidant defences need to operate as a balanced and coordinated system (Halliwell and Gutteridge, 2007). This was evident also in our data on the basis of the correlations between the enzyme activities. GSH showed a positive correlation with GP, which oxidises GSH to its oxidised form GSSG and a negative correlation with GST, which removes hydrogen peroxide (H_2O_2) from the cells through the GSH oxidation (Stephensen et al., 2002; Swiergosz-Kowalewska et al., 2006). CAT, SOD and GP are metalloproteins, which enzymatically detoxify peroxides such as H_2O_2 and O_2^- to less detrimental forms (Gurer and Ercal, 2000). We found a positive correlation between CAT and SOD, which operate together, SOD first transforming superoxide (O_2^-) to hydrogen peroxide, which is then catalysed by CAT to H_2O and molecular oxygen (Finkel and Holbrook, 2000; Pinto et al., 2003). The cooperation between these enzymes is essential—for example if O_2^- are not removed by an SOD, they can partially inhibit CAT generating ferroxycatalase that cannot decompose H_2O_2 fast enough (Halliwell and Gutteridge, 2007). CAT and GP have a similar kind of function in catalysing H_2O_2. CAT however, is more important when the fluctuation of H_2O_2 is high, mainly because of its ability to directly catalyse the decomposition of H_2O_2 to H_2O and O_2. GP instead, deals with lower levels of H_2O_2 generation removing it by using H_2O_2 to oxidise another substrate (GSH) (Halliwell and Gutteridge, 2007). Our results suggest that great tit nestlings in this study may have used GP rather than CAT to remove H_2O_2 from their body, which can be seen as an increased GP activation in the polluted area, whereas the activity of CAT had no variation between the study areas. The reason for that might be the generally low level of pollution and thus also the level of ROS in our study areas, in which case GP may act more efficiently to remove H_2O_2.

4.1.2. Diet, nestling condition and fledging success

We found that the GP activity was higher in polluted sites compared to unpolluted sites, but the difference was rather

related to the nestling body mass than the levels of dietary pollutants. This suggests that the higher GP activity is due to the poorer condition of the nestlings in the polluted area. The nestlings in unpolluted area were heavier and also in better condition compared to nestlings in the polluted area, which most likely indicates differences in diet between the areas. Pollution can reduce some important food sources, such as caterpillars and other forest insects, and hence indirectly affect the breeding performance and survival of the birds (Bengtsson and Rundgren, 1984; Graveland, 1990; Perrins, 1991; Van Noordwijk et al., 1995; Eeva et al., 1997, 2003, 2005). Indirect effects of pollution can be even more common in nature than the direct ones, and may also occur at lower contaminant levels (Eeva et al., 2003). Earlier studies have shown that there are also changes in the quality of diet between polluted and unpolluted areas. In the polluted area, the invertebrate diet of nestlings contains more heavy metals and has a smaller proportion of carotenoid-rich moths and caterpillars, as compared to the unpolluted area (Eeva et al., 2003, 2005, 2008, 2009; Sillanpää et al., 2008). However based on our results, the amount of dietary carotenoids does not explain the variation in GP activity between the study areas. Instead, it is possible that some other factors such as the amount of nutritionally high-quality food (e.g. protein rich food) varies between the study areas, explaining the differences in the nestling body mass as well as brood size between areas. Food supplementation has been shown to increase fledging success and growth rate in nestlings living in polluted areas (Eeva et al., 2003). This also supports our finding that there are better quality nestlings in food-rich environments.

Fledging success was, as expected, better in an unpolluted area than in the polluted area, mainly due to better food supply and high quality food. Also better condition of the nestlings in an unpolluted area can explain the difference in fledging success between the study areas. Increased metal concentration slightly decreased fledging success, which supports our finding of higher mortality and poorer condition of the nestlings in the polluted area as well as the deficiency of good quality diet. Fledging success was also better in those nests, where nestlings were heavy. Interestingly the GP activity was negatively associated with fledging probability and the polluted area showed higher mortality than the unpolluted one. The reason for that may be that the mortality in the polluted area have been continually higher since the hatchling phase, because of limited food supply and other pollution related effects and thus, most of the nestlings with poorer condition have already died leaving smaller broods left in the polluted area, whereas in an unpolluted area the nestling mortality at an early age has been low enabling larger broods. Thus, the nestling mortality is more pronounced during the later parts of the nestling period, when the sibling competition from food and space gets more intense (see also Eeva et al., 1997). This may have increased the GP activity in those nests speeding up the mortality rate as well.

The activity of GST was higher in large broods. This may also be due to an increase within the brood competition for food and space, and thus cause an increased oxidative stress among the nestlings in larger broods. It is known that the brood size can influence many condition related variables, such as growth rate, body condition, body mass, tarsus length and immune response of nestlings (Dijkstra et al., 1990; Mock and Parker, 1997; Alonso-Alvarez et al., 2006). Also some physiological parameters, such as plasma carotenoids and resistance of red blood cells to free radicals have been shown to be affected by an increased nestling competition (Alonso-Alvarez et al., 2006). Gonçalves et al. (2008) showed in mice that enzyme activities also can be related to the social stress (studied by using social confrontation), causing the increase in the GST activity in stressed mice. Our results suggest that this could be the case in birds also, but needs to be confirmed with further studies.

4.2. Oxidative stress, carotenoid treatment and plumage colour

The carotenoid treatment increased the plasma lutein concentration in supplemented birds compared to the control birds, but the response in plasma levels was similar in the two areas. The natural caterpillar availability for great tits has been shown to vary substantially among the years in our study areas and also plasma carotenoid concentrations show large variation between polluted and unpolluted areas between years (Eeva et al., 2005, 2009). The year 2004 seemed to be generally good for carotenoid availability in our study areas.

The carotenoid supplementation has no effect on levels of the oxidative stress in great tit nestlings, mainly due to very low level of oxidative stress. This result suggests that supplemented carotenoids lutein and zeaxanthin, which are the main carotenoids in birds (Tella et al., 2004), are not very effective antioxidants in great tits. If those carotenoids could act as important antioxidants, carotenoid supplemented birds would have been expected to have lower oxidative stress than control birds. However it might also be that in the nestlings of this study, the levels of carotenoids have been high enough to enable an effective defence against ROS, and therefore it was unable to detect any response in the measures of the oxidative stress. Good availability of carotenoids during the study season may therefore have decreased the effect of our lutein manipulation. Sometimes the interactions between different antioxidants (e.g. vitamins C and E vs. carotenoids) might be difficult to detect, because they do not necessarily increase plasma carotenoid concentrations of the study species (Biard et al., 2006). Some earlier studies have shown that carotenoid supplementation has increased vitamin E in birds (Biard et al., 2005, 2006). This and other synergistic interactions between antioxidants, such as the scarcity of any limiting antioxidant can prevent carotenoids to act in an antioxidant role. It is also possible that even though carotenoids would be weak antioxidants in scavenging ROS, they still may be sensitive to the alteration or destruction of their chemical structure or the colour properties caused by ROS (Pérez-Rodríquez, 2009). Carotenoids can, however, still be important antioxidants in cell membranes by protecting phospholipids as well as having a function in an antioxidant defence by participating in the recycling process of vitamin E (Costantini, 2008).

The yellowness of the plumage colour correlated positively with the plasma lutein content and was higher in lutein supplemented group producing brighter yellow plumage in the treated birds. As we did not find any effect between faecal metals and plumage colour, nor any difference between the study areas, we can conclude that the present metal concentrations do not directly affect the variation in the plumage colour (see also Eeva et al., 2008). However, the brood size was positively associated with the plumage colour, suggesting that the good availability of carotenoid-rich food enables bigger broods and also brighter plumage colour in the nestlings. Smaller brood size might be partially due to the decreased food availability, which might have affected the brightness of plumage colour as well. It is possible that there are other factors, such as other antioxidants (e.g. vitamins E and C) than carotenoids, which can be involved to the plumage colour and further to the level of oxidative stress (von Schantz et al., 1999; Hartley and Kennedy, 2004; Bertrand et al., 2006; Pike et al., 2007). Thus, in the future studies, it would be important to measure non-pigmentary antioxidants as well to reveal the relationships between other antioxidants, carotenoids, colouration and oxidative stress.

4.3. Oxidative stress and temperature

The increase in GP activity was related to temperature during the early nestling period, indicating the higher GP activity in

warm temperatures. There are no major differences in temperatures between our study areas. Breeding time temperatures measured inside the empty nest boxes have been 0.6 °C higher in the polluted area compared to an unpolluted area (unpublished data). However, the study area explains $<2\%$ of the variation in temperature, while the temporal changes explain 94% of the variation. The lower GP activity in early summer (beginning of June) might be due to lower breeding time temperatures compared to those in the later breeders (at the end of June). During their early development, nestlings can be considered as poikilothermic, which may be an adaptation to the lack of insulation (plumage). Decreased metabolic rates may have decreased the level of oxidative stress in nestlings experiencing low temperatures. Nestlings of the late breeding females instead have faced the warmer temperature at their nestling time and may therefore have higher metabolic levels and thus higher oxidative stress levels as well compared to nestlings born at an early summer. In an earlier study, Eeva et al. (2003) have shown that the metabolic rate (oxygen consumption) of the great tit nestlings increased with an ambient temperature in their study sites. Temperature and the sampling date correlated positively, supporting the idea of later born nestlings facing more oxidative stress, because of warmer weather later in the breeding season. However to understand the variation in the GP activity throughout the nestling phase, several blood samples should be taken at different time points in development. Norte et al. (2009a) have reported relatively large environmental component of variance of the GP activity in great tit nestlings, suggesting that there is a lot of difference in degree of the oxidative stress among the individuals faced with different rearing conditions, such as food availability, type of food, parasitism and infections in their nestling phase. Environmental component of variance of GP can also be affected by the dietary antioxidant levels (Norte et al., 2009a). The same has been reported in adult great tits, which have shown great seasonal and yearly variation in biochemical, haematological and morphological parameters (Norte et al., 2009b). However, because of the complexity of glutathione metabolism, it needs to be remembered that also some other factors can be related to the level of enzyme activities, and therefore more studies are still needed to confirm our results.

5. Conclusions

Metal pollution did not directly increase the oxidative stress in great tit nestling in our study area, but environmental pollution can indirectly affect the level of the oxidative stress of nestlings mainly because of the deficiency of high quality food in the polluted area. The GP activity increased in the polluted area, suggesting poorer condition (lower body mass) and higher level of the oxidative stress in the nestlings in the polluted area. Dietary carotenoid levels do not seem to explain the variation in the GP activity between the study areas, but the quantity of nutritionally high-quality food instead may vary between the areas causing poor quality nestlings and an increased level of the oxidative stress in contaminated environment. Higher nestling body mass and fledging success in the unpolluted area suggest that nestlings there have received higher quantity/quality food, were in better condition and consequently showed lower GP values than those in the polluted area. Temperature also increased the GP activity, suggesting higher oxidative stress in nestlings born in warmer temperature/later in the breeding season. This may be due to higher metabolic rate of poikilothermic nestlings in the warmer temperature, which can increase the oxidative stress. The plumage colouration did not show any association with faecal metal levels, nor any difference between the study areas. However, the plumage colour intensity increased with an increased carotenoid concentration in both study areas. Carotenoids did not affect oxidative stress levels, suggesting that they were not efficient antioxidants. Based on our results, we can conclude that the exposure levels found in our study area are probably not high enough to cause the oxidative stress in great tit nestlings or the level of oxidative stress appears at very low level. Instead, the GP activity can be used as a sensitive indicator of growth related oxidative stress in great tit nestlings. Clearly, the activity of this enzyme was not directly related to the metal exposure, but more likely to some secondary pollution-related change in the nestling condition. Still, further studies are needed to understand the mechanisms behind the indirect effects of metal exposure to nestling survival and the sensitivity to the oxidative stress.

Conflicts of interest statement

Authors declare no conflicts of interest.

Acknowledgments

We want to thank Saila Sillanpää, Lauri Nikkinen and Jorma Nurmi for the help on collecting data in the field. We also thank Kalle Rainio for valuable comments of the manuscripts. The study was financed by Academy of Finland (Project 8119367) and metal analyses by Boliden Company, Harjavalta, Finland.

References

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B., Sorci, G., 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am. Nat. 164, 651–659.

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., Chastel, O., Sorci, G., 2006. An experimental manipulation of life-history trajectories and resistance to oxidative stress. Evolution 60, 1913–1924.

Andrews, K.G., 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem. Pharmacol. 59, 95–104.

Bel'skii, E.A., Bezel', V.S., Polents, E.A., 1995. Early stages of the nesting period of hollow-nesting birds under conditions of industrial pollution. Russ. J. Ecol.+ 26, 38–43.

Bel'skii, E.A., Lugaśkova, N.V., Karfidova, A.A., 2005. Reproductive parameters of adult birds and morphophysiological characteristics of chicks in the pied flycatcher (*Ficedula hypoleuca* Pall.) in technogenically polluted habitats. Russ. J. Ecol.+ 36, 329–335.

Bengtsson, G., Rundgren, S., 1984. Ground-living invertebrates in metal-polluted forest soils. Ambio 13, 29–33.

Berglund, Å.M.M., Sturve, J., Förlin, L., Nyholm, N.E.I., 2007. Oxidative stress in pied flycatcher (*Ficedula hypoleuca*) nestlings from metal contaminated environments in northern Sweden. Environ. Res. 105, 330–339.

Berglund, Å., 2010. Responses to reduced industrial metal emissions: an ecotoxicological study on pied flycatcher (*Ficedula hypoleuca*, Aves). Doctoral thesis, University of Umeå, Sweden.

Bertrand, S., Faivre, B., Sorci, G., 2006. Do carotenoid-based sexual traits signal the availability of non-pigmentary antioxidants? J. Exp. Biol. 209, 4414–4419.

Biard, C., Surai, P.F., Møller, A.P., 2005. Effects of carotenoid availability during laying on reproduction in the blue tit. Oecologia 144, 32–44.

Biard, C., Surai, P.F., Møller, A.P., 2006. Carotenoid availability in diet and phenotype of blue and great tit nestlings. J. Exp. Biol. 209, 1004–1015.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burger, J., 1993. Metals in avian feathers: bioindicators of environmental pollution. Rev. Environ. Contam. Toxicol. 5, 203–311.

Chew, B.P., Park, J.S., 2004. Carotenoid action on the immune response. J. Nutr. 134, 257S–261S.

Costantini, D., Casagrande, S., De Filippis, S., Brambilla, G., Fanfani, A., Tagliavini, J., Dell'Omo, G., 2006. Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). J. Comp. Physiol. B 176, 329–337.

Costantini, D., 2008. Oxidative stress in ecology and evolution: lessons from avian studies. Ecol. Lett. 11, 1–14.

Costantini, D., Møller, A.P., 2008. Carotenoids are minor antioxidants for birds. Funct. Ecol. 22, 367–370.

Costantini, D., Verhulst, S., 2009. Does high antioxidant capacity indicate low oxidative stress? Funct. Ecol. 23, 506–509.

Dauwe, T., Bervoets, L., Blust, R., Pinxten, R., Eens, M., 2000. Can excrement and feathers of nestling songbirds be used as biomonitors for heavy metal pollution? Arch. Environ. Contam. Toxicol. 39, 541–546.
Dauwe, T., Janssens, E., Bervoets, L., Blust, R., Eens, M., 2004. Relationships between metal concentrations in great tit nestlings and their environment and food. Environ. Pollut. 131, 373–380.
Dijkstra, C., Bult, A., Bijlsma, S., Daan, S., Meijer, T., Zijlstra, M., 1990. Brood size manipulations in the kestrel *Falco tinnunculus*: effects on offspring and parent survival. J. Anim. Ecol. 59, 269–286.
Dmowski, K., 1993. Lead and cadmium contamination of passerine birds (Starlings) during their migration through a zinc smelter area. Acta Ornithol. 28, 1–9.
Dowling, D.K., Simmons, L.W., 2009. Reactive oxygen species as universal constraints in life-history evolution. Proc. R. Soc. London B Biol. 276, 1737–1745.
Eeva, T., 1996. Direct and indirect effects of air pollution on two whole-nesting bird species. Dissertation Series A II tom, 83. University of Turku.
Eeva, T., Lehikoinen, E., 1996. Growth and mortality of nestling great tits (*Parus major*) and pied flycatcher (*Ficedula hypoleuca*) in a heavy metal pollution gradient. Oecologia 108, 631–639.
Eeva, T., Lehikoinen, E., Pohjalainen, T., 1997. Pollution-related variation in food supply and breeding success in two whole-nesting passerines. Ecology 78, 1120–1131.
Eeva, T., Lehikoinen, E., Rönkä, M., 1998. Air pollution fades the plumage of the great tit. Funct. Ecol. 12, 607–612.
Eeva, T., Tanhuanpää, S., Råbergh, C., Airaksinen, S., Nikinmaa, M., Lehikoinen, E., 2000. Biomarkers and fluctuating asymmetry as indicators of pollution-induced stress in two hole-nesting passerines. Funct. Ecol. 14, 235–243.
Eeva, T., Lehikoinen, E., Nikinmaa, M., 2003. Pollution-induced nutritional stress in nestling birds: an experimental study of direct and indirect effects. Ecol. Appl. 13, 1242–1249.
Eeva, T., Lehikoinen, E., 2004. Rich calcium availability diminishes heavy metal toxicity in pied flycatcher. Funct. Ecol. 18, 548–553.
Eeva, T., Ryömä, M., Riihimäki, J., 2005. Pollution-related changes in diets of two insectivorous passerines. Oecologia 145, 629–639.
Eeva, T., Sillanpää, S., Salminen, J.-P., Nikkinen, L., Tuominen, A., Toivonen, E., Pihlaja, K., Lehikoinen, E., 2008. Environmental pollution affects the plumage color of Great tit nestlings through carotenoid availability. EcoHealth 5, 328–337.
Eeva, T., Sillanpää, S., Salminen, J.-P., 2009. The effect of diet quality and quantity on plumage colour and growth of great tit *Parus major* nestlings: a food manipulation experiment along a pollution gradient. J. Avian Biol. 40, 491–499.
Ercal, N., Gurer-Orhan, H., Aykin-Burns, N., 2001. Toxic metals and oxidative stress part I: mechanisms involved in metal induced oxidative damage. Curr. Top. Med. Chem. 1, 529–539.
Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of aging. Nature 408, 239–247.
Garlick, P.B., Davies, M.J., Hearse, D.J., Slater, T.F., 1987. Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. Circ. Res. 61, 757–760.
Geens, A., Dauwe, T., Eens, M., 2009. Does anthropogenic metal pollution affect carotenoid colouration, antioxidative capacity and physiological condition of great tits (*Parus major*)? Comp. Biochem. Physiol. C 150, 155–163.
Gonçalves, L., Dafre, A.L., Gonçalves Carobrez, S., Gasparotto, O.C., 2008. A temporal analysis of the relationship between social stress, humoral immune response and glutathione-related antioxidant defences. Behav. Brain Res. 192, 226–231.
Graveland, J., 1990. Effects of acid precipitation on reproduction in birds. Experientia (Basel) 46, 962–970.
Gurer, H., Ercal, N., 2000. Can antioxidants be beneficial in the treatment of lead poisoning? Free Radical Biol. Med. 29, 927–945.
Halliwell, B., Gutteridge, J., 2007. Free Radicals in Biology and Medicine fourth ed. Oxford University Press, New York.
Hartley, R.C., Kennedy, M.W., 2004. Are carotenoids a red herring in sexual display? Trends Ecol. Evol. 19, 353–354.
Heliövaara, K., Väisänen, R. (Eds.), 1993. Insects and Pollution. CRC Press, Boca Raton, Florida, USA.
Hoffman, D.J., Franson, J.C., Pattee, O.H., Bunck, C.M., Murray, H.C., 1985. Biochemical and hematological effects of lead ingestion in nestling American kestrels (*Falco sparverius*). Comp. Biochem. Phys. C80, 431–439.
Hõrak, P., Ots, I., Vellau, H., Spottiswoode, C., Møller, A.P., 2001. Carotenoid-based plumage coloration reflects hemoparasite infection and local survival in breeding great tits. Oecologia 126, 166–173.
Hõrak, P., Saks, L., Zilmer, M., Karu, U., Zilmer, K., 2007. Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. Am. Nat. 170, 625–635.
Isaksson, C., Oernborg, J., Stephensen, E., Andersson, S., 2005. Plasma glutathione and carotenoid coloration as potential biomarkers of environmental stress in great tits. EcoHealth 2, 138–146.
Isaksson, C., Andersson, S., 2008. Egg yolk carotenoids in relation to habitat and reproductive investment in the great tit *Parus major*. Physiol. Biochem. Zool. 81, 112–118.
Isaksson, C., Sturve, J., Almroth, B.C., Andersson, S., 2009. The impact of urban environment on oxidative damage (TBARS) and antioxidant systems in lungs and liver of great tits, *Parus major*. Environ. Res. 109, 46–50.
Janssens, E., Dauwe, T., Bervoets, L., Eens, M., 2002. Inter- and intraclutch variability in heavy metals in feathers of great tit nestlings (*Parus major*) along a pollution gradient. Arch. Environ. Contam. Toxicol. 43, 323–329.
Janssens, E., Dauwe, T., Pinxten, R., Bervoets, L., Blust, R., Eens, M., 2003. Effects of heavy metal exposure on the condition and health of nestlings of the great tit (*Parus major*), a small songbird species. Environ. Pollut. 126, 267–274.
Kamiński, P., Kurhalyuk, N., Jerzak, L., Kasprzak, M., Tkachenko, H., Klawe, J.J., Szady-Grad, M., Koim, B., Wiśniewska, E., 2009. Ecophysiological determinations of antioxidant enzymes and lipoperoxidation in the blood of white stork *Ciconia ciconia* from Poland. Environ. Res. 109, 29–39.
Kiikkilä, O., 2003. Heavy-metal pollution and remediation of forest soil around the Harjavalta Cu–Ni smelter, in SW Finland. Silva Fenn. 37 (3), 399–415.
Koivula, M.J., Eeva, T., 2010. Metal-related oxidative stress in birds. Environ. Pollut. 158, 2359–2370.
Lozano, G.A., 1994. Carotenoids, parasites, and sexual selection. Oikos 70, 309–311.
Mock, D.W., Parker, G.A., 1997. The Evolution of Sibling Rivalry. Oxford University Press, Oxford.
Monaghan, P., Metcalfe, N.B., Torres, R., 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol. Lett. 12, 75–92.
Montgomerie, R., 2006. Analysing colors. In: Hill, G.E., McGraw, K.J. (Eds.), Bird Coloration. Harvard University Press, Boston, pp. 90–147.
Norte, A.C., Sheldon, B.C., Sousa, J.P., Ramos, J.A., 2009a. Environmental and genetic variation in body condition and blood profile of great tit *Parus major* nestlings. J. Avian Biol. 40, 157–165.
Norte, A.C., Ramos, J.A., Sousa, J.P., Sheldon, B.C., 2009b. Variation of adult great tit *Parus major* body condition and blood parameters in relation to sex, age, year and season. J. Ornithol. 150, 651–660.
Nyholm, N.E.I., 1994. Heavy metal tissue levels, impact on breeding and nestling development in natural populations of pied flycatchers (Aves) in the pollution gradient from a smelter. In: Donker, M., Eijsackers, H., Heimback, F. (Eds.), Ecotoxicology of Soil Organisms. Lewis Publishers, Chelsee, USA, pp. 373–382.
Palozza, P., 1998. Prooxidant actions of carotenoids in biological systems. Nutr. Rev. 56, 257–265.
Partali, V., Liaaen-Jensen, S., Slagsvold, T., Lifjeld, J.T., 1985. Carotenoids in food chain studies—II. The food chain of *Parus* spp. monitored by carotenoid analysis. Comp. Biochem. Physiol. B 82, 767–772.
Pérez-Rodríquez, L., 2009. Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. BioEssays 31, 1116–1126.
Perrins, C.M., 1965. Population fluctuation and clutch-size in the great tit *Parus major* L. J. Anim. Ecol. 34, 601–647.
Perrins, C.M., 1991. Tits and their caterpillar food supply. J. Anim. Ecol. 133, 49–54.
Pike, T.W., Blount, J.D., Lindström, J., Metcalfe, N.B., 2007. Availability of noncarotenoid antioxidants affects the expression of carotenoid-based sexual ornament. Biol. Lett. 3, 353–356.
Pinto, E., Sigaud-Kutner, T.C.S., Leitão, M.A.S., Okamoto, O.S., Morse, D., Colepicolo, P., 2003. Heavy metal-induced oxidative stress in algae. J. Phycol. 39, 1008–1018.
Scheuhammer, A.M., 1987. The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: a review. Environ. Pollut. 46, 263–295.
Sillanpää, S., Salminen, J.-P., Lehikoinen, E., Toivonen, E., Eeva, T., 2008. Carotenoids in a food chain along a pollution gradient. Sci. Total Environ. 406, 247–255.
Stephensen, E., Sturve, J., Förlin, L., 2002. Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. Comp. Biochem. Physiol. C 133, 435–442.
Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biol. Med. 18, 321–336.
Swiergosz-Kowalewska, R., Bednarska, A., Kafel, A., 2006. Glutathione levels and enzyme activity in the tissues of bank vole *Clethrionomys glareolus* chronically exposed to a mixture of metal contaminants. Chemosphere 65, 963.
Tella, J.L., Figuerola, J., Negro, J.J., Blanco, G., Rodríguez-Estrella, R., Forero, M.G., Blázquez, M.C., Green, A.J., Hiraldo, F., 2004. Ecological, morphological and phylogenetic correlates of interspecific variation in plasma carotenoid concentration in birds. J. Evol. Biol. 17, 156–164.
Tummeleht, L., Mägi, M., Kilgas, P., Mänd, R., Hõrak, P., 2006. Antioxidant protection and plasma carotenoids of incubating great tits (*Parus major* L.) in relation to health state and breeding conditions. Comp. Biochem. Physiol. C 144, 166–172.
Villafuerte, R., Negro, J.J., 1998. Digital imaging for colour measurement in ecological research. Ecol. Lett. 1, 151–154.
Van Noordwijk, A.J., McCleery, R.H., Perrins, C.M., 1995. Selection for the timing of great tit breeding in relation to caterpillar growth and temperature. J. Anim. Ecol. 64, 451–458.
von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D., Wittzell, H., 1999. Good genes, oxidative stress and condition-dependent sexual signals. Proc. R. Soc. London B Biol. 266, 1–12.