The Journal of Experimental Biology 213, 2225-2233

doi:10.1242/jeb.042085

Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches

Peeter Hõrak[1,*], Elin Sild[1], Ursel Soomets[2], Tuul Sepp[1] and Kalle Kilk[2]

[1]Department of Zoology, Institute of Ecology and Earth Sciences, The Centre of Excellence FIBIR, Tartu University, Vanemuise 46, 51014 Tartu, Estonia and [2]Department of Biochemistry, Medical Faculty; The Centre of Excellence for Translational Medicine, Tartu University, Ravila 19, 50411 Tartu, Estonia

*Author for correspondence (horak@ut.ee)

Accepted 17 March 2010

SUMMARY

Carotenoid and melanin pigments in the plumage of birds are hypothesized to be sensitive to oxidative stress. We manipulated oxidative status of captive greenfinches (*Carduelis chloris* L.) by the administration of buthionine sulfoximine (BSO), a selective inhibitor of the synthesis of glutathione (GSH), an intracellular antioxidant. Half of the birds in the treated group, as well as in the control group, also received dietary carotenoid (lutein) supplementation. BSO treatment reduced erythrocyte GSH levels and caused oxidative damage as indicated by the increased concentration of plasma malondialdehyde (MDA), an end product of lipid peroxidation. BSO treatment also reduced the brightness (i.e. increased blackness) of the tips of tail feathers grown during the experiment. These results show that a low systemic GSH level is required for development of eumelanin plumage coloration and that such a low GSH level is also potentially dangerous for the organism. Carotenoid supplementation increased plasma carotenoid levels and chroma of the yellow parts of the feathers grown during the experiment. However, carotenoid supplementation did not reduce plasma MDA levels. Manipulation of GSH did not affect plasma carotenoids or carotenoid-based plumage coloration. These findings argue against the antioxidant function of lutein *in vivo* and carotenoid signaling of antioxidant status.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/13/2225/DC1

Key words: buthionine sulfoximine, *Carduelis chloris*, carotenoids, eumelanin, glutathione, malondialdehyde, oxidative damage, oxidative stress, passerine, plumage coloration.

INTRODUCTION

Carotenoids and melanins are the two main pigment classes responsible for the colorful plumage of birds. Carotenoids are responsible for most red, orange and yellow coloration, and melanins are mainly responsible for black (eumelanins) and reddish and brown (pheomelanin) colors. Animals synthesize melanins from the aromatic amino acids phenylalanine and tyrosine but carotenoids must be acquired from diet (reviewed by Hill and McGraw, 2006b; Jawor and Breitwisch, 2003). Amongst both types of pigments, there has been much interest in and controversy over the factors that keep them reliable as signals of individual quality (Griffith et al., 2006; Hill, 2006b; McGraw, 2008). Notably, both types of pigments are claimed to share a common property of being sensitive to oxidative stress (OS) (Griffith et al., 2006; Lozano, 1994; Moreno and Møller, 2006).

OS results from a mismatch between the production of reactive oxygen and nitrogen species (RONS) and the organism's capacity to mitigate their damaging effects. Managing OS is now widely believed to be a major determinant of life histories as virtually all physiological activities generate RONS (Monaghan et al., 2009). Notably, the main insight of animal ecologists to the biology of OS stems from avian studies (Costantini, 2008), not least due to the fact that most conspicuous avian colors are produced by carotenoids, known for their antioxidant properties (Krinsky, 1989; Lozano, 1994; von Schantz et al., 1999). An idea that melanin (and other) pigments, too, can potentially function as antioxidants reached the ecological literature later (Griffith et al., 2006; McGraw, 2005; Moreno and Møller, 2006), and has remained untested in birds (McGraw, 2008). However, melanogenesis is an oxidative process that acts as a potential source of reactive oxygen species inside pigment-forming cells (e.g. Smit et al., 2008).

Thus, apart from the hypothesized antioxidant functions, evolutionary implications of melanin production might be mediated by the oxidative nature of its synthesis. Recently, an interesting new hypothesis suggesting a different link between melanin coloration and oxidative stress has been proposed (Galván and Alonso-Alvarez, 2008; Galván and Solano, 2009) (see also Moreno and Møller, 2006). It claims that because a key intracellular antioxidant – glutathione (GSH) – inhibits eumelanogenesis, eumelanin-based black ornaments signal the bearer's ability to cope with oxidative stress. According to the honest advertisement hypothesis (Zahavi, 1975), only those individuals with low enough levels of GSH for ornament production should manage well the whole of the antioxidant machinery in order to maintain optimal oxidative status. We propose that this idea also has a direct bearing with relation to the hypothesis that the antioxidant function of carotenoids is the responsible mechanism warranting the honesty of carotenoid coloration. If a certain extent of OS is required for a production of eumelanin plumage, and carotenoids are efficient antioxidants, then developing dark black feathers would be incompatible with circulating high levels of carotenoids required for production of intense yellow, orange and red integument coloration, i.e. carotenoid and eumelanin ornaments cannot be maximized together (in terms of pigment deposition), if carotenoids impede depletion of GSH levels in melanocytes. This is an intriguing possibility, which would enable a totally novel insight into the evolution of avian coloration

patterns. However, its proof would crucially depend on further evidence about involvement of OS in the expression of both types of signals. The issue is far from clear because the premise that carotenoids are efficient antioxidants *in vivo*, which was initially warmly greeted by animal ecologists, has now become a matter of debate (e.g. Costantini and Møller, 2008; Hartley and Kennedy, 2004). This implies that the possible antioxidant function of carotenoids requires further clarification by applying proper methods for manipulation of OS and quantification of oxidative damage (Monaghan et al., 2009; Pérez-Rodríguez, 2009).

Evidence, that experimental reduction of systemic GSH indeed increases eumelanic plumage coloration, comes from an experiment with great tit (*Parus major*) nestlings, where blocking GSH synthesis caused the development of wider black breast stripes (Galván and Alonso-Alvarez, 2008). In red-legged partridges (*Alectoris rufa*), chemically induced OS led to increased area of black feather patches (Galván and Alonso-Alvarez, 2009). The latter study also found that although OS induced by diquat-suppressed erythrocyte GSH levels, it, contrary to the prediction, decreased the levels of an independent marker of oxidative damage – thiobarbituric acid reactive substances (TBARS) – in erythrocytes.

In the current study, we address the question about the links between oxidative damage, systemic GSH levels and melanin- and carotenoid-based feather coloration in captive greenfinches. Greenfinches are an excellent model for looking at the mechanisms affecting production and information content of carotenoid-based feather coloration (Aguilera and Amat, 2007; Peters et al., 2008; Saks et al., 2003). Greenfinches are sexually dichromatic, seed-eating passerines that display a variety of carotenoid-based plumage patches, ranging from bright yellow to olive. The color of a contrasting yellow tail patch indicates parasite resistance and immune responsiveness and is strongly associated with plasma lutein concentration and general body condition (reviewed by Peters et al., 2008). Yellow tail and wing feathers have black tips, where melanin probably provides protection from degradation (see McGraw, 2006). These feather tips are also exposed during 'butterfly' display flights. Importantly, dietary carotenoid supplementation of greenfinches has been shown to reduce the systemic oxidative damage (Hõrak et al., 2007), a result seldom demonstrated in adult birds (Costantini and Møller, 2008).

We performed a 2×2 factorial experiment by manipulating dietary carotenoids and systemic GSH levels, and recorded the effects of these treatments upon coloration of black and yellow parts of tail feathers grown during experiment. To clarify how these treatments affect oxidative status of birds, we monitored the changes in erythrocyte GSH, plasma carotenoids, total antioxidant capacity (TAC) and malondialdehyde (MDA) during the experiment. We consider the latter measurement to be particularly informative because MDA, which is produced by peroxidative decomposition of unsaturated lipids, is also mutagenic and cytotoxic and can damage membrane proteins (Halliwell and Gutteridge, 2007). So far, most ecological studies, including the one by Galván and Alonso-Alvarez (Galván and Alonso-Alvarez, 2009), have relied on spectrophotometric detection of TBARS for the assessment of lipid peroxidation in biological material. This method has been severely criticized because of non-specificity and artefactual generation of TBARS during the assay (e.g. Halliwell and Gutteridge, 2007). Quantification of MDA by high-performance liquid chromatography/mass spectrometry (HPLC/MS) is technically more complicated but is devoid of such problems (Andreoli et al., 2003).

Our experiment was set to provide answers to the following questions: (1) can we detect antioxidant effects of dietary carotenoids by applying a HPLC/MS quantification of MDA? (2) Can we reproduce the findings of two previous studies (Galván and Alonso-Alvarez, 2008; Galván and Alonso-Alvarez, 2009) that low systemic GSH levels are required for production of eumelanic plumage coloration in a different avian model? (3) Does the inhibition of GSH synthesis cause oxidative damage? (4) If so, can supplementation of exogenous antioxidants (carotenoids) attenuate damage caused by low GSH? (5) Does OS, imposed by depletion of GSH, affect plasma carotenoids and carotenoid-based plumage coloration? (6) Is there a trade-off in formation of intense yellow and black coloration?

MATERIALS AND METHODS

Male greenfinches were captured in mist-nets at bird feeders in gardens in the city of Tartu, Estonia (58°22′N; 26°43′E) and in the adjacent small town of Elva on 2 to 5 January 2009. The birds were housed indoors in individual cages (27 cm×51 cm×55 cm) with sand-covered floors. Mean temperature in the aviary during the experiment was 15.8±1.8°C (±s.d.) and mean humidity was 52.1±2.9% (±s.d.). The birds were supplied *ad libitum* with sunflower seeds and filtered tap water. Birds were held on the natural daylength cycle on artificial lighting and released into their natural habitat on 5 March. The study was conducted under the license from the Estonian Ministry of the Environment and the experiments comply with the current laws of Estonian Republic.

Experimental protocol

From 6 to 9 January all of the birds were subjected to four-day anticoccidian treatment with Intracox Oral (Interchemie, Castenary, The Netherlands) in order to reduce infection-induced variation in physiological condition of individuals. The birds received 2 ml l^{-1} of the solution containing 25 mg l^{-1} Toltrazuril in their drinking water. Birds were divided into four treatment groups (14–15 birds in each). These groups were set to have similar mean body masses on 9 January and similar age compositions (6–8 first year and older birds in each group). On 10 January (day 1 of the experiment in Fig. 1), half of the birds started receiving 18 μg ml^{-1} of carotenoid solution in their drinking water. Carotenoid supplementation consisted of lutein and zeaxanthin (20:1, w/w), prepared from OroGlo liquid solution of 11 g kg^{-1} xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium). Solutions were freshly prepared each evening using filtered (Brita® Classic; BRITA GmbH, Taunusstein, Germany) tap water and were provided in 50 ml doses in opaque dispensers in order to avoid oxidation of carotenoids. Carotenoid dose was determined on the basis of previous experience (Hõrak et al., 2006) where birds that were supplemented daily with a 10 μg ml^{-1} dose of lutein circulated about 30 μg ml^{-1} carotenoids after 23 days.

To reduce the systemic levels of total GSH, half of the birds in both the carotenoid-supplemented and unsupplemented groups started to receive BSO (Sigma, cat. no. B2640, Sigma Chemical Co., St Louis, MO, USA) injections from 25 January (day 16 of the experiment) onwards. BSO is a synthetic amino acid that irreversibly inhibits gamma-glutamylcysteine synthetase, thereby depleting cells of GSH. The high specificity of BSO makes it a non-toxic compound that produces no other effects than a decrease in GSH levels (Galván and Alonso-Alvarez, 2008). Doses of BSO (50 mg ml^{-1} in 100 μl of sterile isotonic saline) were adjusted from an experiment by Galván and Alonso-Alvarez (Galván and Alonso-Alvarez, 2008), and the lack of detrimental effects was confirmed

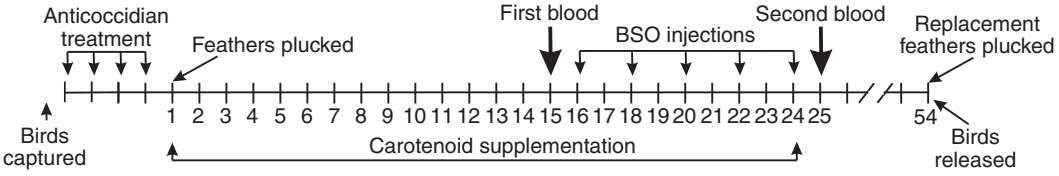

Fig. 1. Course of the experiment. Day 1=10 January. BSO, buthionine sulfoximine.

in a pilot experiment. Birds received five injections into breast muscle on alternate days until 2 February (Fig. 1). Another half of the birds received an injection of the same amount of sterile isotonic saline at the same time.

Birds were blood sampled on 24 January (day 15; i.e. one day before the start of BSO treatment) and on 3 February (10 days later, i.e. one day after the last BSO injection). Blood sampling took place in the morning before the lights were turned on. Other procedures, including maintenance and injections, were performed in the evenings after the lights were turned off. Before blood sampling, body mass was recorded with a precision of 0.1 g. Blood was collected into 300 µl Microvette® (Sarstedt, Nümbrecht, Germany) tubes with lithium heparin. Immediately after blood collection, tubes were placed into a cooled and light-protected box on ice and centrifuged within one hour from sampling for 5 min at 6700 ***g*** to separate plasma from erythrocytes. Plasma and erythrocytes were stored at –80ºC until analyzed.

Biochemical analyses

Concentration of carotenoids was determined spectrophotometrically from 15 µl plasma, diluted in acetone as described by Tummeleht et al. (Tummeleht et al., 2006). Plasma TAC was measured from 5 µl plasma samples according to the method described by Erel (Erel, 2004) with minor modifications as described by Sepp et al. (Sepp et al., 2010). The assay is based on the capacity of antioxidants in the solution to decolorize the $ABTS^+$ [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate)] according to their concentrations and antioxidant capacities. Main contributors to TAC are plasma uric acid and free sulfhydryl groups of proteins (Erel, 2004; Sepp et al., 2010). In a larger dataset of greenfinches sampled in 2009, the correlation between TAC and uric acid was rather low (R=0.31, P<0.0001, N=169), suggesting a major contribution of sulfhydryl groups to variation in TAC. (Uric acid was measured by uricase reaction by a Human GmbH kit, manufactured in Wiesbaden, Germany.)

Total GSH levels in erythrocytes were determined within a week after sampling according to Galván and Alonso-Alvarez (Galván and Alonso-Alvarez, 2008) and Rahman et al. (Rahman et al., 2007) with modifications. The refrigerated erythrocyte pellet was weighed and diluted immediately (1:10 w/v) in 0.1 mol l^{-1} potassium phosphate buffer with 5 mmol l^{-1} EDTA disodium salt (pH 7.5). Homogenate of blood cells was mixed with an equal volume of trichloroacetic acid (Applichem, Darmstadt, Germany; 10% in H_2O) and vortexed three times over 5 s for each bout within a 15 min period as described in Galvàn and Alonso-Alvarez (Galvàn and Alonso-Alvarez, 2008). In the meantime, samples were removed from light and refrigerated to prevent oxidation. The mixture was centrifuged at 3000 ***g*** at 4°C for 15 min. 20 µl of supernatant was transferred into the wells of a microplate. Solutions of DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)], containing 2 mg of reagent (Sigma Chemical Co., cat. no. D-8130) in 3 ml of buffer, and glutathione reductase, containing 40 µl of reagent (Sigma Chemical Co., cat. no. G-3664) in 3 ml buffer, were mixed in equal volumes and 120 µl of this mixture was added to the wells of a microplate. Then, 60 µl of β-NADPH (Sigma Chemical Co., cat. no. NM-7505) solution, containing 2 mg of NADPH in 3 ml of buffer, was added and the absorbance was immediately measured at 412 nm with a Tecan microplate reader (Sunrise, Tecan Austria, Grödig/Salzburg). The measurements were taken every 30 s for 2 min (five readings in total from 0 s to 120 s). The change in absorbance was used to determine the total GSH concentration in erythrocytes by comparing the output with the results from a standard curve generated by the serial dilution of GSH from 13.2 nmol ml^{-1} to 0.825 nmol ml^{-1}. Results are given in µmol per gram of pellet. Repeatability (Lessells and Boag, 1987) of GSH was 0.91 ($F_{9,10}$=22.1, P<0.0001).

Plasma MDA was assessed by HPLC/MS analysis based on a modified protocol from Andreoli et al. (Andreoli et al., 2003). 5 µl of plasma was treated with 20 µl of 1 mol l^{-1} NaOH in order to hydrolyze protein-bound MDA. After 60 min 5 µl of 10 µmol l^{-1} internal standard, 4-hydroxy nonenal-D3 (Cayman Chemicals, Ann Arbor, MI, USA, cat. no. 332201), was added. The solution was acidified with 20 µl of concentrated formic acid. 20 µl of 2 mmol l^{-1} dinitrophenylhydrazone (DNPH) solution in acetonitrile was added and the reaction was left to proceed for 90 min with occasional vortexing. Samples were centrifuged at 21,000 ***g*** for 15 min, the supernatant was transferred into Shimadzu Prominence autosampler vials (Shimadzu, Kyoto, Japan) and 30 µl was injected into a column composed of two PFP 4×3 mm SecurityGuard cartridges (Phenomenex, Torrance, CA, USA). Flow rate was 0.1 ml min^{-1}, gradient rose from 5% acetonitrile in water to 100% acetonitrile within 10 min, followed by 5 min wash at 100% acetonitrile. At the 10th minute the flow was directed into a Q TRAP® 3200 mass spectrometer (Applied Biosystems, Foster City, CA, USA). Ion spray voltage was 4500 V, temperature was 350°C, declustering and collision energies were 30 V. Multiple reaction monitoring m/z values were 235.0→159.0 for MDA and 340.0→167.3 for internal standard. Standard curve was made of commercially available MDA (Fluka, Buchs, Switzerland, cat. no. 63287). Repeatability of plasma MDA concentration was 0.79 ($F_{162,224}$=4.5, P<0.0001).

Plumage coloration

The left outermost tail feather was plucked from all of the birds on 10 January (day 1) and the replacement feather, grown in captivity, was collected on 5 March (day 55). Mass of replacement feathers comprised on average 92% of the mass of wild-grown feathers. Feather color was measured in areas of the visible carotenoid-pigmented and black surfaces of the feather of approximately 1 mm^2 using a spectrophotometer (Ocean Optics USB2000 with Ocean Optics DH2000 lamp). The measurements were taken from the standard positions from the dorsal side of the feather on both vanes, as shown in Fig. 2.

Light was transferred to the feather through a quartz optic fiber (Ocean Optics), reaching the feather at 90 deg. The sampling optic was placed at 45 deg to the surface of the sample and connected to

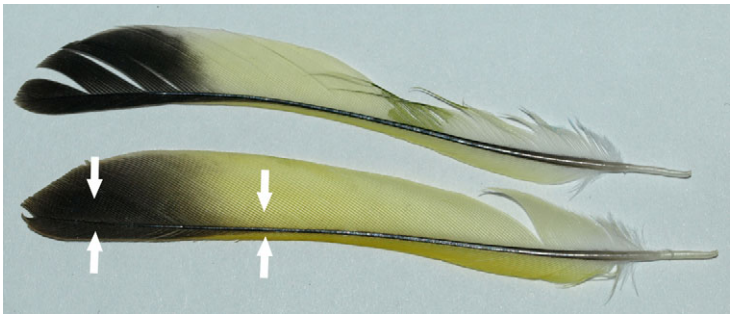

Fig. 2. Outermost tail feathers of male greenfinches. Arrows indicate the approximate positions of color measurements. Lower: wild-grown feather, chroma of the yellow part=0.27; brightness of the black part=3262. Upper: lab-grown feather of the same individual [treated with buthionine sulfoximine (BSO) and not supplemented with carotenoids], chroma of the yellow part=0.18; brightness of the black part=1459. Enlargement the image in Fig. S1 in the supplementary material makes the difference in the blackness clearly visible.

a spectrophotometer by a quartz fiber optic cable. Data from the spectrophotometer were digitalized and passed into a computer with appropriate software (OOIBase, Ocean Optics Inc., Dunedin, FL, USA). The measurements were relative and referred to a standard white reference tile (WS-2) and to the dark. Each measurement provided a measure of percentage reflectance for each 1 nm interval in the range of 400–700 nm. To describe carotenoid-based coloration, we calculated values of chroma according to Endler (Endler, 1990). Chroma is a measure of the 'purity' or 'saturation' of a color, which was calculated as follows:

$$\text{Chroma} = \sqrt{(\text{R} - \text{G})^2 + (\text{Y} - \text{B})^2} \ ,$$

where B=(Q_b/Q_t), G=(Q_g/Q_t), Y=(Q_y/Q_t), R=(Q_r/Q_t). Q_r denotes the summed reflectance in the red area of the reflectance spectrum (between 625 nm and 700 nm), Q_g is the summed reflectance in the green area of the spectrum (between 475 nm and 550 nm), Q_y is the summed reflectance in the yellow area of the spectrum (between 550 nm and 625 nm), Q_b is the summed reflectance in the blue area of the spectrum (between 400 nm and 475 nm) and Q_t (brightness) is the summed reflectance between 400 nm and 700 nm. Chroma of the yellow parts of greenfinch tail feathers reflects their carotenoid content (Saks et al., 2003). Black coloration was characterized on the basis of brightness, which has been shown to correlate strongly with the eumelanin content of feathers: the darker the feathers, the lower the brightness and the higher the melanin content (McGraw et al., 2005b). We ignored UV-reflectance of feathers in our analyses of color because our main interest was obtaining measurements correlating with pigment deposition.

Three consecutive measurements were taken from both the inner and outer vane in yellow and black areas of the feather, removing the probe each time between measurements. The resulting six measurements for both yellow and black areas were averaged as to obtain total feather coloration estimates. Repeatabilities of chroma ranged from 0.90 to 0.96. Repeatabilities of brightness of black ranged from 0.95 to 0.99.

Statistics

Effects of experimental treatments upon changes in physiological parameters were assessed in two-way factorial analyses of covariance (ANCOVAs), including initial trait values as covariates. When analyzing the effect of treatments upon the black coloration of feathers grown during the experiment, we also retained the brightness of wild-grown feathers in a model in order to account for the individually persistent component of this coloration. (Brightness of wild-grown black feathers tips significantly predicted the color of lab-grown feathers; see Results). Chroma of the yellow parts of the wild-grown feathers did not correlate with chroma of the feathers grown in captivity, so in this case simple factorial analysis of variance (ANOVA) was performed. Assumptions for parametric models (normality of residuals, homogeneity of variances and sphericity) were met for all of the variables except plasma carotenoid concentration. However, this variable was affected only by carotenoid supplementation, an effect that was also revealed by a non-parametric test. Sample sizes differ between some analyses due to our inability to collect a sufficient amount of blood from all of the birds. Mean trait values are presented with ±s.d. Age (first year *vs* older) did not affect any of the studied parameters except blackness of wild-grown feathers. All tests are two-tailed with a *P*-level below 0.05 as a criterion for significance. Average values of measured parameters in all experimental groups are presented in supplementary material Table S1.

RESULTS

None of the treatments affected body mass change during the experiment (BSO: $F_{1,58}$=1.3, P=0.26; carotenoids: $F_{1,58}$=0.1, P=0.70) in a model adjusting for pre-treatment body mass. Treatment with BSO caused a 33% decrease in erythrocyte GSH levels while sham-injected birds experienced a 5% increase of GSH (Fig. 3A). Carotenoid treatment had no main effect on the change in erythrocyte GSH levels but the model in Table 1A indicated a significant interaction between the two treatments. Fig. 3A suggests that this was because among BSO-treated birds, those that also received carotenoids experienced the highest drop in GSH levels. On the contrary, among the birds not treated with BSO, carotenoid treatment seemed to increase GSH levels slightly. Further examination by HSD *post hoc* tests, however, did not reveal that carotenoid supplementation had any significant effects on GSH levels within BSO treatment groups (P=0.35 and 0.36).

BSO treatment caused an increase of plasma MDA levels by 14%. MDA levels of sham-injected birds decreased on average by 7% at the same time (Fig. 3B). Carotenoid treatment did not affect the change in plasma MDA (Table 1B). None of the treatments affected the change in plasma TAC levels (Table 1C).

Plasma carotenoid levels increased among supplemented birds by 28% and decreased among unsupplemented birds by 22%. Because carotenoid supplementation started 13 days before first blood sampling, mean plasma carotenoid levels among supplemented birds were 63–78% higher than those of unsupplemented birds during the whole experiment (first sampling: 16.5±16.6 μg ml^{-1} *vs* 6.1±6.2 μg ml^{-1}, second sampling 21.3±13.3 μg ml^{-1} *vs* 4.7±7.5 μg ml^{-1}). BSO treatment or its interaction with carotenoid treatment had no effect on the change in plasma carotenoid concentrations (Table 1D).

Brightness of the black tips of the tail feathers grown during the experiment was 18% lower in BSO-treated birds than in sham-injected control birds (Fig. 3C). Age of birds did not affect the brightness of lab-grown feathers ($F_{1,48}$=0.7, P=0.4). Carotenoid treatment or its interaction with BSO did not affect brightness of the black parts of the feathers in a model adjusting for the brightness of wild-grown feathers (Table 1E). Carotenoid-supplemented birds

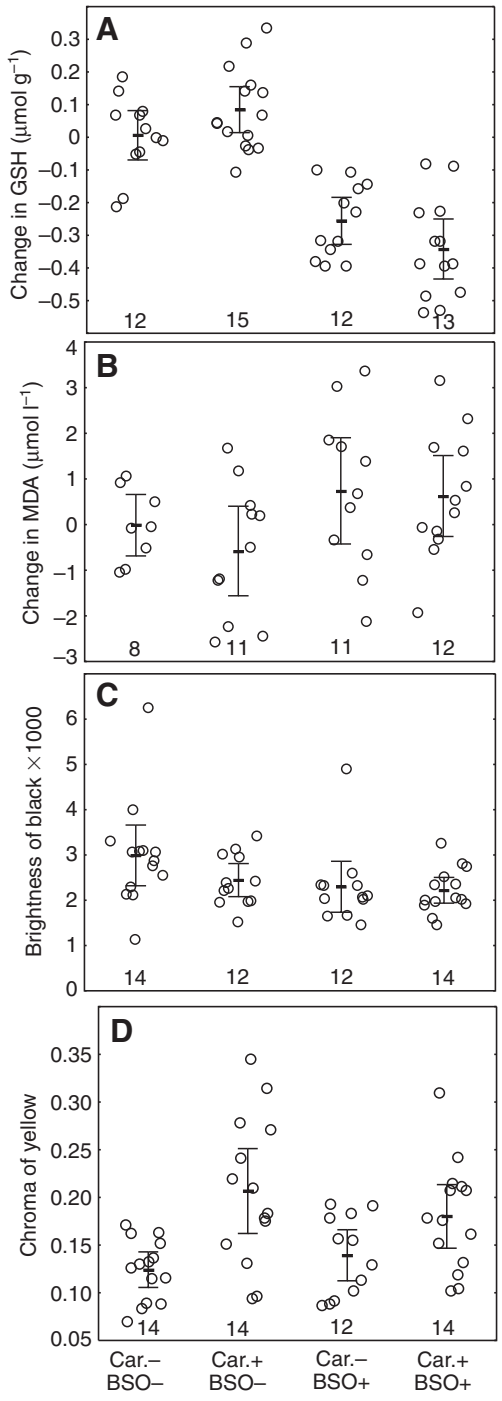

Fig. 3. Effects of administration of carotenoids and buthionine sulfoximine (BSO) upon parameters of oxidative status and plumage coloration of greenfinches. Bars denote means and whiskers denote 95% confidence intervals; individual values are presented as circles. Sample sizes are indicated at the base of bars. See Table 1 for the significance of treatments. Car.+ means carotenoid-supplemented birds, Car.– means birds without carotenoid supplementation, BSO+ means BSO treatment, BSO– means non-BSO treatment.

developed yellow parts of tail feathers with 32% higher values of chroma than unsupplemented birds (Fig. 3D). Treatment with BSO or its interaction with carotenoid treatment had no effect on the chroma of yellow of feathers grown during the experiment (Table 1F).

Because BSO treatment increased plasma MDA levels, we further examined correlations between markers of oxidative status and individual condition. Decrease in erythrocyte GSH levels was associated with an increase in plasma MDA, albeit this correlation was not quite significant (Fig. 4A). Birds that lost more body mass during the experiment also significantly increased their plasma MDA levels (Fig. 4B). Birds with higher plasma MDA levels after treatment also had significantly higher levels of plasma TAC (Fig. 4C) but this correlation was not significant before treatment (R=0.17, P=0.12, N=54). Nor did the change in MDA correlate with change in TAC between treatments (R=–0.08, P=0.60, N=42). Plasma carotenoid levels after treatments correlated with chroma of the yellow parts of the feathers grown during experiment (R=0.67, P<0.0001, N=51) but none of the physiological variables measured was significantly related to blackness of the feather tips grown in the lab (R=–0.25 to +0.19, P=0.08 to 0.83, N=23–47).

DISCUSSION

GSH and oxidative damage

Our results confirm the importance of GSH in the protection against oxidative damage. Treatment with BSO, which suppressed erythrocyte GSH, led to an increase in plasma MDA by 14%. Birds with the highest drop in erythrocyte GSH level generally also had the highest increase in plasma MDA level (Fig. 4A) and lost more weight during the experiment (Fig. 4B). Loss of body mass can be considered one of the most sensitive indicators of physiological condition in avian studies, which suggests that increased plasma MDA levels in our study indeed reflect unfavorable changes in health state. In a study by Galván and Alonso-Alvarez in red-legged partridges, where OS and reduction of erythrocyte GSH was induced by administration of a pro-oxidant molecule (diquat bromide), treated birds showed a significant decline in levels of erythrocyte TBARS, a proxy for measuring lipid peroxidation in a spectrophotometric assay (Galván and Alonso-Alvarez, 2009). The authors interpreted this finding as a possible protective upregulation of antioxidant protection in developing birds but also admit that the specificity of TBARS assay has been questioned. Also, in partridges TBARS levels were measured in erythrocytes, while in the current study, MDA was measured in plasma, which complicates the direct comparison of these two studies.

Effect of BSO treatment on erythrocyte GSH level explained higher proportion of variance than its effect on oxidative damage, as indicated by the comparison of coefficients of partial determination in Table 1 (η^2=0.75 *vs* 0.11). This suggests that the effects of GSH depletion upon lipid peroxidation were largely buffered, possibly by other components of the antioxidant system. Differently from the study of Galván and Alonso-Alvarez (Galván and Alonso-Alvarez, 2008), we did not observe an increase in plasma TAC in response to BSO administration. However, a positive correlation between the values of TAC and MDA emerged after treatment (Fig. 4C), so a possibility of compensatory upregulation of other water-soluble plasma antioxidants cannot be totally excluded. TAC measured in this study by the method of Erel (Erel, 2004) is less strongly correlated with plasma uric acid than other similar measures (see Sepp et al., 2010), so one might expect a rather strong contribution of free –SH groups of proteins (Erel, 2004).

GSH and blackness

BSO treatment decreased brightness of black feather tips. In this respect our experiment successfully replicated findings of two previous studies (Galván and Alonso-Alvarez, 2008; Galván and Alonso-Alvarez, 2009). As a novel aspect, we showed that

Table 1. Effects of carotenoid and BSO administration upon of oxidative status (changes over 10-day period) and plumage coloration of greenfinches

Variable	Predictor	$F_{d.f.}$	P	η^2
(A) Change in GSH	Initial	$23.0_{1,47}$	<0.0001	0.33
	BSO	$144.5_{1,47}$	<0.0001	0.75
	Car.	0.0	0.95	0
	Car. × BSO	$6.6_{1,47}$	0.01	0.12
(B) Change in MDA	Initial	$33.8_{1,37}$	<0.0001	0.48
	BSO	$4.6_{1,37}$	0.04	0.11
	Car.	$0.1_{1,37}$	0.73	0.00
	Car. × BSO	$0.9_{1,37}$	0.34	0.02
(C) Change in TAC	Initial	$36.4_{1,44}$	<0.0001	0.45
	BSO	$0.1_{1,44}$	0.74	0.00
	Car.	$0.1_{1,44}$	0.73	0.00
	Car. × BSO	1.2	0.29	0.03
(D) Change in carotenoids	Initial	$38.9_{1,49}$	<0.0001	0.44
	BSO	$1.3_{1,49}$	0.26	0.03
	Car.	$19.2_{1,49}$	0.0001	0.28
	Car. × BSO	$1.2_{1,49}$	0.28	0.02
(E) Reflectance of black	Initial	$8.3_{1,47}$	0.006	0.15
	BSO	$3.4_{1,47}$	0.05	0.08
	Car.	$2.0_{1,47}$	0.16	0.04
	Car. × BSO	$0.4_{1,47}$	0.53	0.01
(F) Chroma of yellow	BSO	$0.1_{1.50}$	0.70	0.00
	Car.	$16.6_{1.50}$	0.0001	0.25
	Car. × BSO	$1.9_{1.50}$	0.17	0.04

η^2 stands for coefficients of partial determination, describing the proportion of total variation attributable to the factor, partialling out other factors from the total non-error variation. Car., carotenoids; Initial, trait values at first sampling except for reflectance of black where it denotes the colour of wild-grown feathers; BSO, buthionine sulfoximine; GSH, glutathione; MDA, malondialdehyde; TAC, total antioxidant capacity.

blocking GSH synthesis affected brightness of color, while the previous studies have measured the area of eumelanin patches. Although the generality of these findings waits for further research from other taxa, they raise the question of why and how the links between GSH and eumelanin production evolved. An original hypothesis (Galván and Alonso-Alvarez, 2008; Galván and Solano, 2009) proposed that eumelanin-based ornaments signal the bearers' ability to cope with OS. Thus, according to the honest signaling principle, because GSH depletion implies costs in terms of increased antioxidant mobilization to compensate for GSH scarcity, individuals of low quality will be deterred from investing in a eumelanin signal because the costs (i.e. higher mortality or increased senescence due to OS) are not balanced by the benefits (i.e. increased reproductive success).

In terms of signaling theory, however, it makes a difference whether low GSH levels constitute a cost or a physiological constraint. In the latter case, eumelanin coloration would fit the definition of an 'indicator trait', reflecting individual quality reliably by design (Hasson, 1997; Maynard Smith and Harper, 2003; Vanhooydonck et al., 2007). Further, in addition to the signaling, melanin pigments in birds, as well as in insects and mammals, have many utilitarian functions such as free radical scavenging, cation chelation, immunomodulation, tissue strengthening, photoprotection, thermoregulation, crypsis and camouflage (Ducrest et al., 2008; Griffith et al., 2006; McGraw, 2006; McGraw, 2008). Utilitarian traits enhance an individual's performance, and thereby they can (but do not necessarily need) signal about quality (Zahavi, 2007). For instance, the aim of crypsis is opposite to that of signaling but it might still require investment into eumelanin coloration. Things can get more complicated assuming that even within an individual, some eumelanin traits may function as signals (or signal amplifiers) while others are entirely utilitarian. Furthermore, eumelanins mostly co-occur with pheomelanins in the same feathers (McGraw, 2006), and pheomelanin production seems to require higher GSH levels (Galván and Alonso-Alvarez, 2009; Galván and Solano, 2009). All this suggests further interesting directions for the research of animal coloration and signal evolution. For instance, as suggested by Zahavi (Zahavi, 2007), signal selection and utilitarian selection can interact, so that a signal trait can acquire a new utilitarian function (and possibly *vice versa*).

Ultimately, the question about the information content of eumelanin coloration hangs on the physiological impact of low GSH levels for an organism. GSH is a major endogenous antioxidant, participating directly in the neutralization of free radicals, as well as recycling exogenous antioxidants such as vitamins C and E. GSH is a cofactor in glutathione peroxidases, which reduce H_2O_2 and peroxidized fatty acid residues. GSH is involved in detoxification of xenobiotics and in many aspects of immunomodulation. GSH also plays a fundamental role in numerous metabolic and biochemical reactions such as DNA synthesis and repair, regulation of apoptosis, protein synthesis, prostaglandin synthesis, amino acid transport and enzyme activation (Halliwell and Gutteridge, 2007; Perricone et al., 2009; Sen and Packer, 2000; Wu et al., 2004). GSH also serves as a cysteine reservoir, which, among other functions, has been shown to support muscle and feather growth in birds (Murphy and King, 1990).

Carotenoids

Contrary to our expectations, carotenoid supplementation did not affect plasma MDA levels. Neither did manipulation of GSH affect blood carotenoid levels. In a previous experiment with captive greenfinches, carotenoid supplementation reduced the plasma levels of lipid peroxidation products by 24% (Hõrak et al., 2007). In that study, however, we relied on a spectrophotometric assay (LPO-586 by Oxis Research, Foster City, CA, USA) to assess oxidative damage. This assay measures both MDA and 4-hydroxyalkenals, unlike the HPLC/MS method used in the current experiment, which measures only MDA.

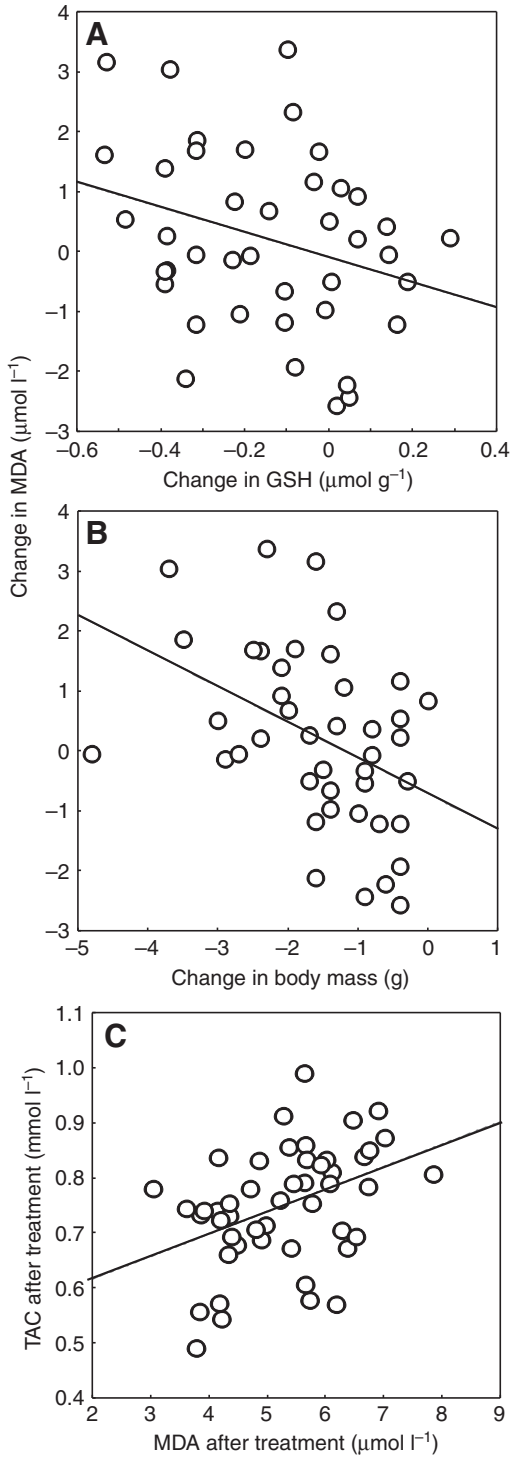

Fig. 4. Correlations between indices of oxidative status and individual condition. Lines are linear regression lines. (A) Change in erythrocyte glutathione (GSH) *vs* change in plasma malondialdehyde (MDA) (R=–0.29, P=0.065, N=41). (B) Change in body mass *vs* change in plasma MDA (R=–0.41, P=0.006, N=42). Changes occurred over 10-day period between first and second blood sampling. (C) Plasma MDA levels after treatment *vs* plasma total antioxidant capacity (TAC) after treatment (R=0.41, P=0.004, N=48).

In any case, the current study fails to support the antioxidant function of carotenoids *in vivo*, by using highly specific and reliable assay for assessment of the oxidative damage. At first sight, this result seems compatible with the review by Costantini and Møller (Costantini and Møller, 2008), finding little support for antioxidant function of carotenoids in birds. It should be noted, however, that none of the papers reviewed by Costantini and Møller (Costantini and Møller, 2008) used specific HPLC-based assays for the assessment of the lipid peroxidation products (a direct measure of damage). Furthermore, approximately half of the papers reviewed have relied on tests measuring the effects of water-soluble antioxidants only (TEAC or AOP-490 tests). The plasma antioxidant capacity measured by such assays correlates very strongly with plasma uric acid levels (Sepp et al., 2010), which could be an indication of incidental amino acid catabolism rather than regulated antioxidant protection (Cohen et al., 2007). So far, antioxidant function of carotenoids in birds has been demonstrated mainly in yolks, embryos and hatchlings (Blount et al., 2002; McGraw et al., 2005a; Surai, 2002), and in few studies of adults (Alonso-Alvarez et al., 2008; Alonso-Alvarez et al., 2009). All of these studies have relied on spectrophotometric measurement of TBARS, which has been criticized, mainly because of the artefactual TBARS generation during the assay (reviewed by Halliwell and Gutteridge, 2007). Independent support for the potential antioxidant function of carotenoids in birds originates from works relying on measurement of oxidized GSH (Alonso-Alvarez et al., 2010) or HPLC-based MDA assay (Mougeot et al., 2009). We conclude that there is no sufficient information for either supporting or rejecting the possible antioxidant function of carotenoids in adult birds. This question has to be addressed in further experiments involving manipulation and assessment of different antioxidant systems (e.g. dietary *vs* endogenous, enzymatic *vs* micromolecular). Furthermore, there is perhaps no reason to expect that the interactions of carotenoids with these various components should be identical (see Pérez-Rodríguez, 2009).

As expected, carotenoid supplementation enhanced the chroma of tail feathers regrown during the experiment and there was a strong correlation (R=0.67) between plasma carotenoid levels and feather chroma. Both results confirm the previous findings in greenfinches (Karu et al., 2008; Peters et al., 2008) and many other birds (Hill and McGraw, 2006a). Manipulation of GSH had no effect on the yellow coloration of tail feathers, which argues against the hypothesis that carotenoids indicate the abundance of other, non-colorful antioxidants (Hartley and Kennedy, 2004). Similarly, induction of oxidative stress by pro-oxidant paraquat did not affect the carotenoid mobilization or yellow pigmentation in great tits (Isaksson and Andersson, 2008). However, strong correlation between plasma carotenoid content and chroma of the yellow indicates a very clear link with the physiological state of an individual, as long as this state covaries with plasma carotenoids. Ample evidence indicates that parasites, malnutrition, poor health state and sometimes immune activation deplete plasma and integumentary carotenoids in birds (reviewed by Hill, 2006a). The mechanisms related to food absorption are perhaps the clearest ones – carotenoids (unlike amino acid precursors of melanins) are endocytosed by intestinal epithelium and this process directly depends on the integrity of that epithelium (e.g. Jawor and Breitwisch, 2003).

The immunostimulatory properties of carotenoids have also been hypothesized to play a relevant role in the allocation trade-offs, as diseased individuals must allocate greater amounts of carotenoids to immune function, and these are therefore not available for ornament expression (Lozano, 1994). However, the concept of ‘immunocompetence’ is vague itself because maximum immune responses are not necessarily optimal and activation of some

components of the immune system may downregulate others (e.g. Graham et al., 2005). We thus conclude that despite decades of intense work, the question what exactly is being signaled by carotenoid ornaments is still very much open.

Conclusions

This study provided support for the effect of oxidative stress on expression of eumelanic traits as depletion of systemic GSH produced an increase in plumage melanization and of oxidative damage. However, we found no evidence that dietary carotenoid supplementation (which affected yellow plumage coloration) had reduced oxidative damage. Neither did manipulation of GSH affect plasma carotenoids or carotenoid-based plumage coloration. These findings argue against the antioxidant function of lutein *in vivo* and carotenoid signaling of antioxidant status. The proposed hypothesis about oxidative stress-mediated trade-off between formation of intense yellow and black coloration could not thus be supported.

LIST OF ABBREVIATIONS

BSO	buthionine sulfoximine
GSH	glutathione
HPLC/MS	high-performance liquid chromatography/mass spectrometry
MDA	malondialdehyde
OS	oxidative stress
RONS	reactive oxygen and nitrogen species
TAC	total antioxidant capacity
TBARS	thiobarbituric acid reactive substances

ACKNOWLEDGEMENTS

We thank Lauri Saks, Ulvi Karu, Marju Männiste and Richard Meitern for the help with blood sampling, bird maintenance and measuring feather color. George Lozano and two anonymous reviewers provided constructive criticism on the ms. Stefaan Van Dyck (Kemin Agrifoods Europe) kindly donated OroGlo carotenoid supplement. The study was financed by Estonian Science Foundation (grants # 7737 to P.H. and # 7494 to K.K.), the Estonian Ministry of Education and Science (target-financing project # 0180004s09) and by the European Union through the European Regional Development Fund (Centres of Excellence FIBIR and Translational Medicine).

REFERENCES

Aguilera, E. and Amat, J. (2007). Carotenoids, immune response and the expression of sexual ornaments in male greenfinches (*Carduelis chloris*). *Naturwissenschaften* **94**, 895-902.

Alonso-Alvarez, C., Perez-Rodriguez, L., Mateo, R., Chastel, O. and Vinuela, J. (2008). The oxidation handicap hypothesis and the carotenoid allocation trade-off. *J. Evol. Biol.* **21**, 1789-1797.

Alonso-Alvarez, C., Perez-Rodriguez, L., Garcia, J. T. and Vinuela, J. (2009). Testosterone-mediated trade-offs in the old age: a new approach to the immunocompetence handicap and carotenoid-based sexual signalling. *Proc. R. Soc. Lond. B. Biol. Sci.* **276**, 2093-2101.

Alonso-Alvarez, C., Perez-Rodriguez, L., Garcia Jesus, T., Vinuela, J. and Mateo, R. (2010). Age and breeding effort as sources of individual variability in oxidative stress markers in a bird species. *Physiol. Biochem. Zool.* **83**, 110-118.

Andreoli, R., Manini, P., Corradi, M., Mutti, A. and Niessen, W. M. A. (2003). Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **17**, 637-645.

Blount, J. D., Surai, P. F., Houston, D. C. and Møller, A. P. (2002). Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. *Funct. Ecol.* **16**, 445-453.

Cohen, A., Klasing, K. and Ricklefs, R. (2007). Measuring circulating antioxidants in wild birds. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **147**, 110-121.

Costantini, D. (2008). Oxidative stress in ecology and evolution: lessons from avian studies. *Ecol. Lett.* **11**, 1238-1251.

Costantini, D. and Møller, A. P. (2008). Carotenoids are minor antioxidants for birds. *Funct. Ecol.* **22**, 367-370.

Ducrest, A. L., Keller, L. and Roulin, A. (2008). Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends Ecol. Evol.* **23**, 502-510.

Endler, J. A. (1990). On the measurement and classification of colour in studies of animal colour patterns. *Biol. J. Linn. Soc.* **41**, 315-352.

Erel, O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.* **37**, 277-285.

Galván, I. and Alonso-Alvarez, C. (2008). An intracellular antioxidant determines the expression of a melanin-based signal in a bird. *PLoS ONE* **3**, e3335.

Galván, I. and Alonso-Alvarez, C. (2009). The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. *Proc. R. Soc. Lond. B. Biol. Sci.* **276**, 3089-3097.

Galván, I. and Solano, F. (2009). The evolution of eu- and pheomelanic traits may respond to an economy of pigments related to environmental oxidative stress. *Pigment Cell Melanoma Res.* **22**, 339-342.

Graham, A. L., Allen, J. E. and Read, A. F. (2005). Evolutionary causes and consequences of immunopathology. *Annu. Rev. Ecol. Evol. Syst.* **36**, 373-397.

Griffith, S. C., Parker, T. H. and Olson, V. A. (2006). Melanin-versus carotenoid-based sexual signals: is the difference really so black and red? *Anim. Behav.* **71**, 749-763.

Halliwell, B. and Gutteridge, J. M. C. (2007). *Free Radicals in Biology and Medicine.* Oxford: Oxford University Press.

Hartley, R. C. and Kennedy, M. W. (2004). Are carotenoids a red herring in sexual display? *Trends Ecol. Evol.* **19**, 353-354.

Hasson, O. (1997). Towards a general theory of biological signaling. *J. Theor. Biol.* **185**, 139-156.

Hill, G. E. (2006a). Environmental regulation of ornamental coloration. In *Bird Coloration. Volume I. Mechanisms and Measurements*, vol. 1 (ed. G. E. Hill and K. J. McGraw), pp. 507-560. Cambridge, MA: Harvard University Press.

Hill, G. E. (2006b). Female mate choice for ornamental colouration. In *Bird Colouration. Function and Evolution*, vol. II (ed. G. E. Hill and K. J. McGraw), pp. 137-200. Cambridge, MA: Harvard University Press.

Hill, G. E. and McGraw, K. J. (2006a). *Bird Coloration. Volume I. Mechanisms and Measurements.* Cambridge, MA: Harvard University Press.

Hill, G. E. and McGraw, K. J. (2006b). *Bird Coloration. Volume II. Function and Evolution.* Cambridge, MA: Harvard University Press.

Hõrak, P., Zilmer, M., Saks, L., Ots, I., Karu, U. and Zilmer, K. (2006). Antioxidant protection, carotenoids, and the costs of immune challenge in greenfinches. *J. Exp. Biol.* **209**, 4329-4338.

Hõrak, P., Saks, L., Zilmer, M., Karu, U. and Zilmer, K. (2007). Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. *Am. Nat.* **170**, 625-635.

Isaksson, C. and Andersson, S. (2008). Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. *Proc. R. Soc. Lond. B. Biol. Sci.* **275**, 309-314.

Jawor, J. M. and Breitwisch, R. (2003). Melanin ornaments, honesty, and sexual selection. *Auk* **120**, 249-265.

Karu, U., Saks, L. and Hõrak, P. (2008). Carotenoid-based plumage coloration is not affected by vitamin E supplementation in male greenfinches. *Ecol. Res.* **23**, 931-935.

Krinsky, N. I. (1989). Antioxidant functions of carotenoids. *Free Radic. Biol. Med.* **7**, 617-635.

Lessells, C. M. and Boag, P. T. (1987). Unrepeatable repeatabilities: a common mistake. *Auk* **104**, 116-121.

Lozano, G. A. (1994). Carotenoids, parasites and sexual selection. *Oikos* **70**, 309-311.

Maynard Smith, J. and Harper, D. G. C. (2003). *Animal Signals.* Oxford: Oxford University Press.

McGraw, K. J. (2005). The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? *Anim. Behav.* **69**, 757-764.

McGraw, K. J. (2006). Mechanics of melanin-based coloration. In *Bird Coloration. Mechanisms and Measurements*, vol. I (ed. G. E. Hill and K. J. McGraw), pp. 243-294. Cambridge, MA: Harvard University Press.

McGraw, K. J. (2008). An update on the honesty of melanin-based color signals in birds. *Pigment Cell Melanoma Res.* **21**, 133-138.

McGraw, K. J., Adkins-Regan, E. and Parker, R. S. (2005a). Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. *Naturwissenschaften* **92**, 375-380.

McGraw, K. J., Safran, R. J. and Wakamatsu, K. (2005b). How feather colour reflects its melanin content. *Funct. Ecol.* **19**, 816-821.

Monaghan, P., Metcalfe, N. B. and Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**, 75-92.

Moreno, J. and Møller, A. (2006). Are melanin ornaments signals of antioxidant and immune capacity in birds. *Acta Zool. Sin.* **52**, 202-208.

Mougeot, F., Martínez-Padilla, J., Webster, L. M. I., Blount, J. D., Pérez-Rodríguez, L. and Piertney, S. B. (2009). Honest sexual signalling mediated by parasite and testosterone effects on oxidative balance. *Proc. R. Soc. Lond. B. Biol. Sci.* **276**, 1093-1100.

Murphy, M. E. and King, J. R. (1990). Diurnal changes in tissue glutathione and protein pools of molting white-crowned sparrows-the influence of photoperiod and feeding schedule. *Physiol. Zool.* **63**, 1118-1140.

Pérez-Rodríguez, L. (2009). Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. *BioEssays* **31**, 1116-1126.

Perricone, C., de Carolis, C. and Perricone, R. (2009). Glutathione: a key player in autoimmunity. *Autoimmun. Rev.* **8**, 697-701.

Peters, A., Delhey, K., Andersson, S., van Noordwijk, H. and Forschler, M. I. (2008). Condition-dependence of multiple carotenoid-based plumage traits: an experimental study. *Funct. Ecol.* **22**, 831-839.

Rahman, I., Kode, A. and Biswas, S. K. (2007). Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat. Protoc.* **1**, 3159-3165.

Saks, L., McGraw, K. and Hõrak, P. (2003). How feather colour reflects its carotenoid content. *Funct. Ecol.* **17**, 555-561.

Sen, C. and Packer, L. (2000). Thiol homeostasis and supplements in physical exercise. *Am. J. Clin. Nutr.* **72**, 653S.

Sepp, T., Sild, E. and Hõrak, P. (2010). Hematological condition indices in greenfinches: effects of captivity and diurnal variation. *Physiol. Biochem. Zool.* **83**, 276-282.

Smit, N. P. M., Nieuwpoort, F. A., Marrot, L., Out, C., Poorthuis, B., van Pelt, H., Meunier, J. R. and Pavel, S. (2008). Increased melanogenesis is a risk factor for

oxidative DNA damage-study on cultured melanocytes and atypical nevus cells. *Photochem. Photobiol.* **84**, 550-555.
Surai, P. F. (2002). *Natural Antioxidants in Avian Nutrition and Reproduction.* Nottingham: Nottingham University Press.
Tummeleht, L., Mägi, M., Kilgas, P., Mänd, R. and 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.
Vanhooydonck, B., Van Damme, R., Herrel, A. and Irschick, D. J. (2007). A performance based approach to distinguish indices from handicaps in sexual selection studies. *Funct. Ecol.* **21**, 645-652.
von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. and Wittzell, H. (1999). Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. Lond. B. Biol. Sci.* **266**, 1-12.
Wu, G., Fang, Y.-Z., Yang, S., Lupton, J. R. and Turner, N. D. (2004). Glutathione metabolism and its implications for health. *J. Nutr.* **134**, 489-492.
Zahavi, A. (1975). Mate selection: a selection for a handicap. *J. Theor. Biol.* **53**, 205-214.
Zahavi, A. (2007). Sexual selection, signal selection and the handicap principle. In *Reproductive Biology and Phylogeny of Birds: Sexual Selection, Behavior, Conservation, Embryology and Genetics*, Part B (ed. B. M. N. Jamieson), pp. 143-159. Enfield, New Hampshire England: Science Publishers.

Table S1. Parameter oxidative status and plumage coloration of greenfinches in four different experimental groups at first and second blood sampling

	First sampling				Second sampling			
	Mean	95% CI	Min.-max.	*N*	Mean	95% CI	Min.-max.	*N*
CAR–								
BSO–								
GSH (μmol g^{-1})	0.95	0.881–1.02	0.76–1.21	13	0.93	0.86–1.01	0.71– 1.10	14
MDA (μmol l^{-1})	5.10	4.34–5.87	3.68–6.89	11	5.01	4.51–5.50	3.77–6.18	12
TAC (mmol l^{-1})	0.66	0.58–0.73	0.37–0.84	13	0.74	0.66–0.81	0.49–0.916	13
Carotenoids (μg ml^{-1})	5.05	1.55–8.54	0.00–22.34	13	4.60	0.58–8.61	0.00–27.67	15
Brightness of black	3298.7	2733–3865	1851–4925	14	2990	2319–3661	1142–6266	14
Chroma of yellow	0.25	0.23–0.27	0.18–0.29	14	0.12	0.11–0.14	0.07–0.17	14
CAR+								
BSO–								
GSH (μmol g^{-1})	0.92	0.84–1.01	0.66–1.23	15	1.01	0.95–1.07	0.80–1.24	17
MDA (μmol l^{-1})	5.08	4.56–5.60	3.74–6.43	14	4.79	4.05–5.54	3.04–6.91	13
TAC (mmol l^{-1})	0.74	0.67–0.81	0.50–0.98	16	0.74	0.67–0.80	0.54–0.99	15
Carotenoids (μg ml^{-1})	19.82	9.42–30.22	0.00–60.98	16	24.79	16.68–32.90	0.00–53.10	16
Brightness of black	3175	2689–3662	2338–5131	14	2445	2079–2810	1523–4322	12
Chroma of yellow	0.25	0.23–0.26	0.19–0.28	14	0.21	0.16–0.25	0.09–0.35	14
CAR–								
BSO+								
GSH (μmol g^{-1})	0.89	0.83–0.95	0.73–1.04	15	0.64	0.58–0.69	0.51–0.81	13
MDA (μmol l^{-1})	5.08	4.34–5.81	3.09–8.16	15	5.53	4.66–6.41	3.92–7.85	12
TAC (mmol l^{-1})	0.74	0.65–0.84	0.32–1.15	15	0.75	0.69–0.81	0.57–0.87	12
Carotenoids (μg ml^{-1})	6.92	3.39–10.46	0.00–26.82	15	4.87	0.34–9.40	0.00–21.54	13
Brightness of black	3009	2705–3313	2234–3679	12	2298	1736–2860	1459–4906	12
Chroma of yellow	0.26	0.23–0.28	0.19–0.34	12	0.14	0.11–0.17	0.09–0.19	12
CAR+								
BSO+								
GSH (μmol g^{-1})	0.93	0.86–0.99	0.65–1.11	15	0.57	0.50–0.63	0.44–0.86	13
MDA (μmol l^{-1})	5.14	4.38–5.89	2.05–7.66	14	5.63	5.22–6.04	4.38–6.51	13
TAC (mmol l^{-1})	0.84	0.76–0.92	0.51–1.06	15	0.78	0.71–0.84	0.58–0.91	12
Carotenoids (μg ml^{-1})	15.98	8.19–23.77	0.00–41.79	15	16.12	10.66–21.57	0.00–29.80	15
Brightness of black	3211	2770–3625	2072–4385	14	2220	1936–2504	1469–3264	14
Chroma of yellow	0.26	0.24–0.28	0.21–0.31	14	0.18	0.15–0.21	0.10–0.31	14

BSO stands for buthionine sulfoximine. GSH stands for glutathione. MDA stands for malondialdehyde. TAC stands for total antioxidant capacity.