Behavioral Ecology
doi:10.1093/beheco/arr116
Advance Access publication 11 July 2011

Original Article

Reproductive effort transiently reduces antioxidant capacity in a wild bird

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Oxidative stress has been suggested as a proximate cost of reproduction and hence as a major constraint in the evolution of life histories, and it is therefore thought that antioxidants alleviate the effects of reproductive effort on oxidative stress. Furthermore, carotenoid-based ornaments have been proposed to mirror male ability to resist oxidative stress. Using a full-factorial experimental design in a natural population of great tits *Parus major*, we manipulated brood size and supplemented the male parent with either carotenoids or a placebo. We then assessed antioxidant capacity via a measure of whole blood resistance to a free radical attack during the nestling rearing period. Males of enlarged broods showed impaired antioxidant capacity 5 days after the brood size manipulation. However, 13 days after manipulation, they had their antioxidant capacity restored, an effect that may be due to the development of compensatory antioxidant mechanisms or due to reduced investment in the current reproduction in favor of future survival and reproduction. Carotenoid supplementation did not affect male antioxidant capacity nor was the interaction with the brood manipulation significant. Males with stronger carotenoid-based plumage colors did not show higher antioxidant capacity 5 days after the brood size manipulation, but after 13 days, the relationship was highly significant. This study on a natural population shows that larger brood size can induce a transient decrease in antioxidant capacity. It also supports the hypothesis that carotenoid-based plumage may signal male ability to resist oxidative stress, particularly during the energetically demanding nestling rearing period. *Key words:* antioxidant capacity, brood size manipulation, carotenoid-based coloration, carotenoids, oxidative stress, sexual selection. *[Behav Ecol 22:1218–1226 (2011)]*

INTRODUCTION

Metabolic rate steeply increases during reproduction (Bryant 1997; Moreno et al. 1997; Verhulst and Tinbergen 1997; Nilsson 2002). This increase in metabolism results in higher production of free radicals, mainly reactive oxygen species (ROS) (Bejma and Ji 1999; Finkel and Holbrook 2000; Alessio et al. 2000; Balaban et al. 2005), which may cause substantial exposure to oxidative stress, defined as a disturbance in the balance between pro-oxidants and antioxidants in favor of the former (Sies 1991), and thus determines the rate at which oxidative damage is generated (Costantini and Verhulst 2009). Such an excess of ROS has dramatic physiological consequences because ROS provoke oxidative damage to all biological molecules: proteins, lipids, carbohydrates, and DNA (Gerschman et al. 1954; Halliwell and Gutteringe 2007). Therefore, oxidative stress has been hypothesized to be a major proximate cost of reproduction (Salmon et al. 2001). Hence, individual ability to circumvent oxidative stress may underlie the trade-off between current and future reproduction and survival, oxidative stress thus potentially playing a pivotal role in the evolution of life histories (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009). Oxidative stress as a potential cost to reproduction was demonstrated with experiments on captive animals, where manipulating reproductive effort was shown to deplete circulating antioxidants and/or increased susceptibility to oxidative stress (Salmon et al. 2001; Alonso-Alvarez, Bertrand, Devevey, Prost et al. 2004). However, experiments investigating this hypothesis on natural populations of vertebrates are lacking (Metcalfe and Alonso-Alvarez 2010).

To scavenge ROS and mitigate their negative consequences, organisms use enzymatic molecules such as superoxide dismutase (SOD), catalase or glutathione reductase (GSR) and peroxydase (GPX), and nonenzymatic antioxidants such as vitamins A, C, E, or carotenoids (Beckman and Ames 1998; Finkel and Holbrook 2000). Carotenoids are fat-soluble natural pigments (Goodwin 1984; Surai 2002) that cannot be synthesized de novo by animals and thus must be ingested with food. In addition, carotenoids are a limiting resource in nature, and individuals thus face a trade-off in their allocation to different functions (von Schantz et al. 1999; Møller et al. 2000; Blount, Surai, Houston, et al. 2002; Alonso-Alvarez, Bertrand, Devevey, Gaillard, et al. 2004). The in vitro antioxidant properties of carotenoids are well known (Vershinin 1999; Krinsky 2001; Kiokias and Gordon 2004), and studies have shown that carotenoids may alleviate the cost of immune activity and reproduction in terms of reduced oxidative damage or enhanced resistance to oxidative stress (Bertrand, Alonso-Alvarez, et al. 2006; Hõrak et al. 2006, 2007). However, their in vivo antioxidant properties are currently challenged (Hartley and Kennedy 2004; Costantini et al. 2007; Isaksson and Andersson 2008; Larcombe et al. 2010), and a recent meta-analysis in birds suggests that carotenoids are minor antioxidants in this taxonomic group (Costantini and Møller 2008). In addition to their potential role as antioxidants, carotenoids, and particularly β-carotene have also been shown to be immunoenhancers (Bendich 1989). Carotenoids and their derivative products (retinoids, vitamin A) up- and downregulate immune

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Received 13 January 2011; revised 20 April 2011; accepted 24 May 2011.

activity. On the one hand, they may enhance the expression of genes involved in immune cell proliferation and differentiation (Bendich 1989; Chew and Park 2004; Hartley and Kennedy 2004). On the other hand, they may keep the inflammatory response (fever, anemia, reduced activity [i.e., sickness behavior], production of reactive oxygen and nitrogen species) below deleterious levels (Bendich 1989; Koutsos et al. 2003; Chew and Park 2004; Hartley and Kennedy 2004). However, their function as regulator of the immune system may also be related to their antioxidant properties (Pérez-Rodríguez 2009). All together, these physiological functions may also explain why supplementing breeding individuals with carotenoids increases their reproductive effort (Blount, Surai, Nager et al. 2002; Bertrand, Alonso-Alvarez, et al. 2006; Berthouly 2007; Biard et al. 2007; Helfenstein, Berthouly, et al. 2008). Thus, it is still poorly known how much carotenoid actually contribute to an individual's antioxidant system, allowing it to invest more in reproduction, particularly in wild species on which few studies have been conducted so far. Aviary and field studies may differ considerably because aviary birds typically are fed ad libitum, whereas free-living birds must acquire all their food on their own. Furthermore, metabolic rates of free-living animals may be elevated because of foraging but also due to antipredator behavior and intra- and interspecific competition for limited resources.

Carotenoids are pigments used in the coloration of the plumage of birds, and numerous studies show female preferences for males with the most carotenoid-colored attributes (Hill 1991; Andersson 1994; Olson and Owens 1998; Badyaev and Hill 2000; Andersson and Prager 2006). Carotenoid-based ornamentations have been shown to be honest condition-dependent traits (Badyaev and Hill 2000), potentially reflecting health status of males via foraging ability (Hill 1992) or ability to cope with parasites (Lozano 1994). Carotenoid-based colorful traits may also advertise their bearer's ability to resist oxidative stress (von Schantz et al. 1999; Bertrand, Faivre, et al. 2006; Pike, Blount, Lindstrom, et al. 2007) through at least 2 nonmutually exclusive mechanisms. Carotenoid-based colors may reflect the capacity of a male to cope with the trade-off between using carotenoids either as antioxidants or as pigments ("trade-off" hypothesis; von Schantz et al. 1999) or may reflect the ability of a male to acquire, absorb, and metabolize carotenoids (Hill 1991; Møller et al. 2000) as well as other more potent or interacting colorless dietary antioxidants, which may in turn protect carotenoid from oxidation and make them accessible for coloration or antioxidant processes ("protection" hypothesis; Bertrand, Faivre, et al. 2006; Hartley and Kennedy 2004; Catoni et al. 2008).

Enlarging brood size of great tits increases workload and reproductive effort of males, as illustrated by several studies, including our study population (Lessells 1993; Richner et al. 1995; Verhulst and Tinbergen 1997; Sanz and Tinbergen 1999; Neuenschwander et al. 2003), thus potentially subjecting males to an increased transient oxidative stress (Clarkson and Thompson 2000; Alonso-Alvarez, Bertrand, Devevey, Prost, et al. 2004; Ristow et al. 2009). In this study, we increased brood size and supplemented males with physiological doses of carotenoids. Nestling condition on Day 15 posthatch (well correlated with first-year survival and therefore used as a measure of offspring fitness: Tinbergen and Boerlijst 1990; Heeb et al. 1999) was strongly affected by the brood size manipulation but positively influenced by the carotenoid supplementation of males (Losdat et al. 2010). Here, we investigated in a natural population of great tits whether 1) increasing brood size reduces male antioxidant capacity and 2) carotenoids contribute to male antioxidant capacity. Male great tits *Parus major* exhibit a sexually dichromatic carotenoid-based breast coloration known to be condition dependent (Fitze et al. 2003; Tschirren et al. 2003; Evans et al. 2010) and to show large between-individual variation related to individual quality (Peters et al. 2008). We thus further predicted that 3) carotenoid-based plumage coloration reflects male antioxidant capacity.

MATERIALS AND METHODS

This experiment was conducted during spring 2008 in a natural population of great tits breeding in nest-boxes in a forest near Bern, Switzerland (46°7′N, 7°8′E). Nest-boxes were regularly visited from the beginning of the breeding season to finally determine in 65 nests the start of egg laying and hatching dates. Females were not captured to minimize disturbance to the nest.

Brood size manipulation

Two days posthatch (Day 0 = hatching date), by flipping a coin all nests were randomly assigned to be either augmented with 2 nestlings or kept as original size. We experimentally manipulated brood size of half the nests ($n = 31$) and we visited all other nests but left them unchanged ($n = 34$). The additional nestlings used for increasing brood size came from 31 other nests of the same population that were not included in this study.

Carotenoid supplementation

Seven days posthatch, all 65 males were caught at the nest using electronic traps triggered from a distance using a remote control and randomly assigned to be carotenoid supplemented or to receive a placebo. This resulted in a fully crossed fully randomized design with respect to both brood size manipulation and carotenoid supplementation. Males were force-fed with either one fresh living *Calliphora* spp. larva coated with a blend of corn oil, lutein, zexanthin, and β-carotene (carotenoid supplemented) or with one larva coated with corn oil only (placebo). Carotenoids were provided in the relative proportions found in the natural diet of great tits (80%, 3%, and 17%, respectively; Partali et al. 1987). Males were captured again on Day 11 and the carotenoid supplementation was repeated. On each occasion, we provided 4 times the daily amount of carotenoids that males obtain naturally (Helfenstein, Losdat, et al. 2008), that is, 0.29 mg of total carotenoids per supplementation occasion. Because carotenoids are lipid-soluble antioxidants that birds can store in their liver (Surai 2002) and subsequently use over several days (Inouye 1999; McGraw 2006), our mode of supplementation aimed at doubling, on average, the daily intake of carotenoids over the entire experimental period. Two males could not be recaptured on Day 11 and therefore only received a single dose of carotenoids. Because their inclusion in the data set does not qualitatively change the results, they were kept in the data set.

Morphological measurements

In total, 65 males were captured 7 days posthatch. We measured their body mass (±0.1 g) and the length of their left wing (±0.5 mm) and right tarsus (±0.05 mm). Recent studies have shown that small wintering birds optimize their fat reserves and body mass (Lilliendahl 1997; Krams et al. 2009) and thus body mass corrected for body size does not necessarily reflect body condition during winter. In our study, however, all individuals were trapped during the nestling period, and none of the birds measured showed any fat reserves (score 0 according to Kaiser (1993). Hence, all males in this study could only rely on their muscle mass (e.g., pectoral

Table 1
Initial parameters with regard to the experimental groups on Day 7 and Day 15

	Day 7			Day 15				
	Enlarged	Control	$F_{df}\|P$	Enlarged/ placebo	Enlarged/ supplemented	Control/ placebo	Control/ supplemented	$F_{df}\|P$
N	34	31	—	9	10	12	7	—
Laying date	61.4 ± 3.4	62.2 ± 7.2	$F_{1,62} = 0.32$, $P = 0.57$	59.9 ± 3.7	60.0 ± 3.7	59.9 ± 6.7	63.6 ± 5.9	All $F_{1,34} < 1.03$, $P > 0.32$
Clutch size	8.27 ± 1.53	8.26 ± 1.44	$F_{1,62} < 0.01$, $P = 0.99$	8.7 ± 1.1	8.0 ± 2.0	8.3 ± 1.4	8.4 ± 2.3	All $F_{1,34} < 0.01$, $P > 0.45$
Brood size	7.7 ± 1.80	7.88 ± 1.34	$F_{1,62} = 0.21$, $P = 0.65$	7.8 ± 1.9	7.5 ± 1.9	8.2 ± 1.2	8.3 ± 1.4	All $F_{1,34} < 1.06$, $P > 0.31$
Tarsus length	20.52 ± 0.67	20.75 ± 0.75	$F_{1,62} = 1.62$, $P = 0.21$	20.78 ± 0.46	20.39 ± 0.86	20.41 ± 0.76	20.78 ± 0.66	All $F_{1,34} < 2.68$, $P > 0.11$
Wing length	77.4 ± 2.1	77.7 ± 2.4	$F_{1,62} = 0.18$, $P = 0.67$	78.2 ± 1.99	77.5 ± 1.95	78.1 ± 2.31	77.7 ± 3.10	All $F_{1,34} < 0.54$, $P > 0.47$
Breast color (PCA scores)	0.40 ± 1.33	−0.40 ± 1.77	$F_{1,62} = 4.10$, $P = 0.047$	1.00 ± 1.55	0.39 ± 1.10	−0.87 ± 2.16	−0.02 ± 1.49	$F_{1,34}$ (brood enlarg.) = 4.98, $P = 0.03$

Values are means ± standard deviation. F and P are extracted from analyses of variance including the brood size manipulation for values on Day 7 and the brood size manipulation in interaction with the carotenoid supplementation for values on Day 15. PCA, principal component analysis.

muscles) as possible energy reserves to face any energetic challenge. Because male tarsus length was randomized with regard to treatments (Table 1), variation in residual mass will reflect variation in condition, and thus, within-individual change in body mass should also reliably reflect individual change in condition.

We took a blood sample from the brachial vein and used 7 µl to analyze their red blood cell antioxidant capacity. We could not collect blood samples large enough to extract plasmatic carotenoids. However, the positive effect of a carotenoid-rich diet on carotenoid plasmatic levels is well documented (Surai 2002; Isaksson et al. 2007) and we would expect the same pattern here.

On Day 15 posthatch, 38 of the 65 males were recaptured, weighed, and blood sampled. Because not all males were recaptured, we checked for a potential sample bias but found no difference between recaptured and nonrecaptured males with respect to their mate's laying date, initial brood size, brood size manipulation, carotenoid treatment or male traits measured on Day 7 (body size, breast color, body mass, resistance to oxidative stress) (all $F < 2.57$ and $P > 0.11$).

Breast color

On Day 7 posthatch, we recorded reflectance spectra of the yellow breast plumage of males on 4 different patches, that is, on both sides of the keel on the furcula and on both sides of the belly. We took 2 reflectance readings per patch to assess repeatability, removing the probe from the plumage between each measure. Spectral measures were made using a USB4000 spectrophotometer, an FCR-7UV200-2-ME bifurcated reflectance probe with a 200-µm fiber core diameter, and a deuterium–halogen/tungsten light source (DH-2000-BAL, UV-VIS-NIR; Ocean Optics Inc., The Netherlands). Measurements were made following the recommendations by Andersson and Prager (2006). The tip of the probe was fitted with a black PVC cylinder to standardize measuring distance and exclude ambient light. The probe was held perpendicular to the plumage surface. Each measurement was the average of 4 scans with a 100 ms integration time and was calculated relative to a diffuse reflectance standard (WS-1, Ocean Optics Inc.). The spectrophotometer was calibrated before each individual was measured. S.L. took all measurements.

Color vision in birds depends on 4 types of single cones that are sensitive to very short (VS), short (S), medium (M), and long (L) wavelengths (Hart et al. 2000). Recently, physiological models of color vision have been developed (Vorobyev et al. 1998; Endler and Mielke 2005), which allow to describe a colored trait in the eye of a conspecific taking into account the spectral sensitivity of the retinal cones, the transmittance properties of the ocular media, and the ambient light irradiance spectrum (Endler and Mielke 2005). Using the SPEC package (Hadfield and Owens 2006), we computed 4 cone quantum catches that quantify the amount of light captured by each of the avian single cones (Vorobyev et al. 1998). We used data on cone spectral sensitivities and ocular media transmittance for the blue tit *Cyanistes caeruleus* (Hart et al. 2000). Passerine species that are sensitive to UV wavelength show little variation in their spectral sensitivity and using the average cone-capture function for 11 UV-type species provided by Endler and Mielke (2005) did not qualitatively change the results. We used the forest shade irradiance spectrum (Endler 1993) because our great tit population breeds in forest. The cone catches were standardized using the von Kries algorithm to account for color constancy (Hart et al. 2000). Each cone quantum catch was divided by the sum of all 4, and relative cone quantum catch were then transformed according to Kelber et al. (2003). This transformation projects

the tetrahedral avian visual space into a 3D space. Each color measure is now defined by a set of Euclidean *x*, *y*, *z* coordinates where higher values of *x* represent greater stimulation of the L cones and lower stimulation of the M cones, higher *y* values represent greater stimulation of the S cones, and higher values of *z* represent greater stimulation of the VS cones. The *x*, *y*, and *z* coordinates were repeatable over the 2 repetitions per patch (intraclass correlation coefficient: *x*: $r = 0.43$, $P < 0.001$; *y*: $r = 0.49$, $P < 0.001$; *z*: $r = 0.63$, $P < 0.001$). Measurements were thus averaged per repetition and further per patch to characterize each individual. We then conducted a principal component analysis on the correlation matrix to characterize the variation in color in this Euclidian space (Peters et al. 2008). The first principal component explained 91.4% of the variance and was positively correlated with *x* ($r = 0.57$) and negatively correlated with *y* ($r = -0.57$) and *z* ($r = -0.59$). Therefore, PCA1 (first principal component), hereafter referred to as "breast color," reflects a variable ranking males from those with more yellow (positive scores) to less yellow plumage (more "green-blue") (negative scores). This estimate of breast color was highly positively correlated with carotenoid chroma ($r = 0.87$, $P < 0.001$, $n = 65$), a known measure of the amount of pigment deposited in the feathers (Saks et al. 2003), computed as $(R_{700} - R_{450})/R_{\text{average}}$.

Antioxidant capacity

We assessed male antioxidant capacity using the KRL test (Brevet Spiral V02023, Couternon, France; http://www.nutriteck.com/sunyatakrl.html) adapted to physiological parameters of birds (osmolarity and temperature) (Alonso-Alvarez, Bertrand, Devevey, Gaillard, et al. 2004; Alonso-Alvarez, Bertrand, Devevey, Prost, et al. 2004). This assay reflects the current availability of total antioxidant defenses (enzymatic and nonenzymatic; Lesgards et al. 2002) as well as the past oxidative insults experienced by red blood cells (Esterbauer and Ramos 1996; Brzezinska-Slebodzinska 2001) and also indicates the rates of lipid peroxidation in the erythrocyte membrane (Zou et al. 2001). This assay thus likely integrates both a measure of the oxidative damage undergone by blood cells in a recent past and a measure of antioxidant capacity, that is, the current ability of red blood cells to resist oxidative stress owing to their current susceptibility to oxidative stress. Briefly, 7 µl of whole blood were immediately diluted in 255.5 µl of KRL buffer (150 mM 120 mM Cl^-, 6 mM K^+, 24 mM HCO_3^-, 2 mM Ca^{2+}, 340 mOsM, pH 7.4) and stored at 4 °C before analysis 6.2 ± 3 h after blood collection. The interval before performing the analyses did not influence the results ($F_{1,52} = 0.07$, $P = 0.80$). We loaded 80 µl of KRL-diluted whole blood into wells of a 96-well microplate. We subsequently added to each well 136 µl of a 150 mM solution of 2,2′-azobis-(amidinopropane) hydrochloride (AAPH; a free radical generator), that is, 646 mg of AAPH diluted in 20 ml of KRL buffer (Rojas Wahl et al. 1998). The microplate was subsequently read with a microplate reader spectrophotometer (PowerWave XS reader, Witec AG, Switzerland) at 40 °C. The rate of hemolysis was determined by the change in optical density measured at 540 nm (Alonso-Alvarez, Bertrand, Devevey, Gaillard, et al. 2004; Alonso-Alvarez, Bertrand, Devevey, Prost, et al. 2004; Bertrand, Alonso-Alvarez, et al. 2006). We used the initial optical density as an estimation of the haematocrit, which is likely to influence the rate of hemolysis. We further entered this parameter as a covariate in all the models involving resistance to oxidative stress to control for it.

Statistical analyses

We used linear models with maximum likelihood estimation to analyze 1) body mass on Day 7, 2) body mass change assessed as the difference in body mass between Day 7 and Day 15, 3) log-transformed (to normalize data) antioxidant capacity on Day 7, and 4) log-transformed resistance to oxidative stress on Day 15 posthatch. Although antioxidant capacity on Days 7 and 15 are not independent, we performed separate analyses because we had different predictions for the 2 occasions and also because conducting a repeated model on the 2 measures or using the difference between the 2 measures was less parsimonious while giving qualitatively similar results (see RESULTS).

Models for data on Day 7 included brood size manipulation, breast color, brood size at hatching, and their 2-way interactions. Models for data on Day 15 included carotenoid treatment, brood size manipulation, breast color, brood size at hatching, and their 2-way interactions. There was a slight imbalance between groups in breast plumage color (i.e., males with increased brood size had more colored breast; $F_{1,62} = 4.10$, $P = 0.047$; brood-enlarged males, mean: 0.44, range: −1.74 to 3.18; control males, mean: −0.40, range: −4.94 to 2.90, Table 1). Because the intensity of carotenoid-based coloration is positively related to individual quality in the present great tit population (Fitze et al. 2003; Tschirren et al. 2003), the bias in our sample renders the analyses and results more conservative. Additionally, we statistically corrected for this difference in coloration between groups by including breast color in all models. However, the bias precludes a meaningful interpretation of the interaction between brood size manipulation and breast color, which was thus excluded from the models. In all models, laying date was further entered as a covariate to correct for potential seasonal effects. To check the fit of the models, residuals were tested for normality and homoscedasticity and further plotted against the predicted values. Models were reduced using a backward stepwise elimination procedure based on Akaike information criteria (AICs), retaining the model with the lowest AIC. Tests were 2-tailed with a significance level set to $\alpha = 0.05$. The analyses were performed with R version 2.11.1 (R Development Core Team 2008). Sample sizes vary slightly from one analysis to another because not all individuals could be measured for all traits.

RESULTS

On Day 7, males did not significantly differ in their tarsus length and wing length, and their mates' laying date, clutch size, and initial brood size with regard to the brood size manipulation (Table 1). On Day 15, there was also no significant difference among the 4 experimental groups regarding the same traits (Table 1). Body mass on Day 7 was not significantly affected by brood size manipulation and not statistically correlated with initial brood size or breast coloration (all $F < 2.17$, $P > 0.14$). Antioxidant capacity on Day 7 was significantly affected by the brood size manipulation (Table 2) with males caring for enlarged broods having lower antioxidant capacity (Figure 1).

Change in male body mass from Day 7 to Day 15 posthatch was significantly affected by both brood size manipulation and carotenoid supplementation (Table 3). Carotenoid-supplemented males lost less body mass (−0.20 ± 0.11 g, $n = 17$; from a mean of 17.6 ± 0.9 g on Day 7 to a mean of 17.4 ± 0.9 g on Day 15) than placebo males (−0.54 ± 0.08 g, $n = 20$; from a mean of 18.1 ± 0.7 g on Day 7 to a mean of 17.6 ± 0.8 g on Day 15, i.e., an average of 3% of their body mass, Figure 2). Males caring for enlarged broods lost less body mass than controls (enlarged broods: −0.21 ± 0.08, $n = 19$; control broods: −0.55 ± 0.11, $n = 19$, Figure 2).

Antioxidant capacity on Day 15 was positively correlated with yellow breast color (corrected for experimental groups, Table 4, Figure 3). Finally, male antioxidant capacity was not

Table 2

Linear model testing for an effect of the brood size manipulation on male antioxidant capacity 7 days posthatch, that is, after 5 days of treatment

Effect	Estimate ± standard error	F_{df}	P
Intercept	0.45 ± 0.38	—	—
Brood size manipulation[a]	**1.03 ± 0.45**	$\mathbf{10.32_{1,50}}$	**0.002**
Laying date	−0.01 ± 0.01	$2.24_{1,48}$	0.14
Haematocrit	−0.03 ± 0.07	$0.14._{1,49}$	0.71
Initial brood size	**0.08 ± 0.05**	$\mathbf{1.23_{1,50}}$	**0.27**
Breast color	−0.01 ± 0.03	$0.21_{1,47}$	0.65
Initial brood size × breast color	−0.01 ± 0.02	$0.16_{1,46}$	0.69
Brood size manipulation* × *initial brood size[b]	−0.16 ± 0.06	$8.01_{1,50}$	0.006

The model was reduced using a backward stepwise procedure based on AIC (AIC initial = 112.66; AIC final = 107.70). Terms retained in the final model are highlighted in bold. F and P values of terms not retained in the final model are those immediately prior removal.

[a] Relative to the control brood group.

[b] Significance of the effect of brood size manipulation if removing this interaction (spuriously produced by values from brood sizes 4 and 5 that are not represented in the control group): $F_{1,\ 52} = 8.32$, $P = 0.006$, AIC = 112.86.

statistically affected by the carotenoid supplementation (Table 4). Performing a repeated model or using the difference between the 2 trapping occasions led to qualitatively similar results (significant interaction between day of capture and breast color; $F_{1,25} = 7.13$, $P = 0.013$ and significant effect of the breast color; $F_{1,22} = 0.018$, respectively).

DISCUSSION

Subjecting breeding males to experimentally increased brood size led to a reduction in antioxidant capacity, confirming oxidative stress as a proximate cost of reproduction. Furthermore, male carotenoid-based coloration covaried with the antioxidant capacity toward the end of the rearing period. However, supplementing males with extra carotenoids did not enhance antioxidant capacity, although it had a positive effect on body condition.

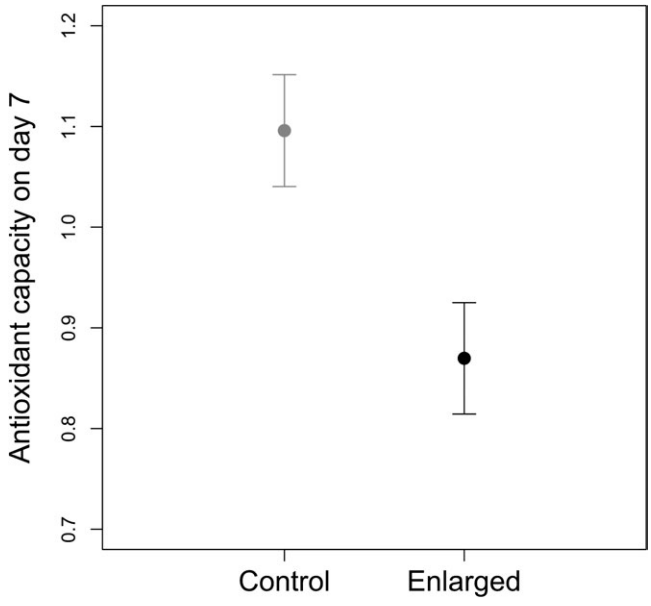

Figure 1
Log-transformed whole blood resistance to a controlled free radical attack (minutes) on Day 7 posthatch (mean ± standard error) in relation to the brood size manipulation.

Given that experimentally enlarging brood size is known from previous studies to increase workload of males (our study population: Neuenschwander et al. 2003; and others: Lessells 1993; Richner et al. 1995; Sanz and Tinbergen 1999), this result suggests that reproductive effort increases susceptibility to oxidative stress. Moreover, during these same 5 days of increased workload, males have been shown to exhibit higher oxidative damage in sperm (Helfenstein et al. 2010), thus strongly suggesting that reproductive effort indeed generates oxidative stress. This result corroborates studies conducted on captive animals (Alonso-Alvarez, Bertrand, Devevey, Prost, et al. 2004; Wiersma et al. 2004; Alonso-Alvarez et al. 2006), which support oxidative stress as a cost of reproduction (Alonso-Alvarez, Bertrand, Devevey, Prost, et al. 2004; Costantini 2008).

However, 13 days after the brood size manipulation, we could no longer detect an effect on male antioxidant capacity, and these males also lost less body mass than control ones between Days 7 and 15. Three scenarios may explain these results. First, it may reflect the fact that after a period of acute oxidative stress (ca. 5 days), and within the course of our experiment (13 days), males have developed compensatory antioxidant mechanisms, such as enhanced antioxidant enzyme synthesis (Monaghan et al. 2009), which restored their ability to overcome oxidative stress. Increased physical activity is indeed likely to enhance free radical production but also to trigger an upregulation of antioxidant enzyme synthesis and/or an increase in the activity of mitochondrial un-coupling proteins, which may quickly regulate the internal homeostasis (Clarkson and Thompson 2000; Leeuwenburgh and Heinecke 2001). Under this scenario, the oxidative cost of reproduction may not be paid during the current reproductive event but later in terms of survival and/or future reproduction if upregulation of antioxidant capacity entails metabolic cost or the depletion of antioxidant reserves.

Second, given the strong negative effect of the brood size enlargement on the offspring (Losdat et al. 2010), males facing the risk of intense oxidative stress with potentially large detrimental effects on survival may have strategically reduced investment in current reproduction to preserve their body condition and antioxidant capacity, thus favoring future survival and/or reproduction (de Ayala et al. 2006). The effect on future reproduction is, however, unknown to us. Nonetheless, this result suggests that oxidative stress may not only be increased by

Table 3
Linear model testing for an effect of brood size manipulation and carotenoid supplementation on difference in male body mass from Day 7 to Day 15 posthatch

Effect	Estimate ± standard error	F_{df}	P
Intercept	−0.65 ± 0.11	—	—
Carotenoid treatment[a]	**0.28 ± 0.14**	$\mathbf{4.11_{1,34}}$	**0.05**
Brood size manipulation[b]	**0.29 ± 0.14**	$\mathbf{4.39_{1,34}}$	**0.04**
Laying date	0.0007 ± 0.01	$0.003_{1,32}$	0.95
Initial brood size	0.06 ± 0.04	$2.13_{1,33}$	0.15
Breast color	−0.01 ± 0.05	$0.07_{1,31}$	0.79
Carotenoid treatment × initial brood size[a]	−0.12 ± 0.09	$1.82_{1,29}$	0.19
Initial brood size × breast color	−0.04 ± 0.03	$2.20_{1,30}$	0.15
Carotenoid treatment × brood size manipulation[c]	0.19 ± 0.30	$0.38_{1,27}$	0.54
Brood size manipulation × initial brood size[b]	−0.03 ± 0.13	$0.05_{1,26}$	0.82
Carotenoid treatment × breast color[a]	−0.06 ± 0.09	$0.35_{1,28}$	0.56

The model was reduced using a backward stepwise procedure based on AIC (AIC initial = −53.39; AIC final = −62.70). Terms retained in the final model are highlighted in bold. F and P values of terms not retained in the final model are those immediately prior removal.

[a] Relative to the placebo group.

[b] Relative to the control brood group.

[c] Relative to the placebo group and the control brood group.

reproductive effort but may also constrain reproduction (Metcalfe and Alonso-Alvarez 2010), and thus acts as a proximate mechanism mediating the trade-off between current and future reproduction (Alonso-Alvarez, Bertrand, Devevey, Prost, et al. 2004; Costantini 2008; Monaghan et al. 2009).

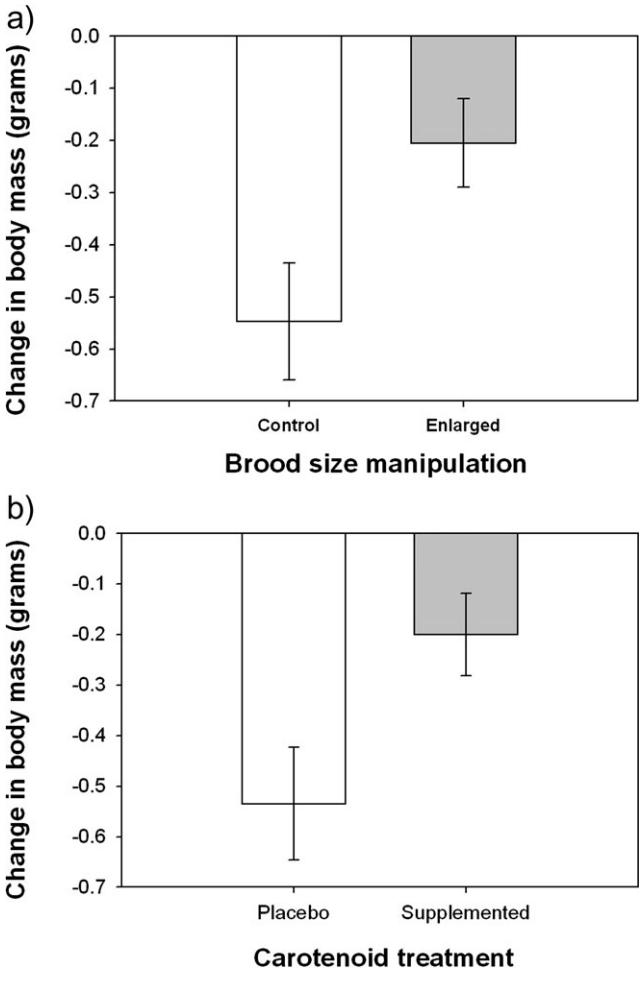

Figure 2
Change in body mass (mean ± standard error) from Day 7 to 15 posthatch in relation to a) brood size manipulation and b) carotenoid supplementation.

Third, because males caring for enlarged broods were more colored and thus presumably of better quality (Fitze et al. 2003; Tschirren et al. 2003), the positive effect on male body mass could reflect either a better ability of males to maintain body condition throughout reproduction or an increased maternal investment in brood care (Burley 1988). However, because nestlings were in poorer condition in the enlarged group (Losdat et al. 2010), this possibility seems unlikely.

We did not detect a direct contribution of carotenoid supplementation on male antioxidant capacity. This result adds to a growing number of studies investigating the antioxidant properties of carotenoids that produced contradictory results, suggesting that in vivo antioxidant properties of carotenoids may be overestimated (Costantini and Møller 2008). However, supplemented males lost less body mass from Day 7 to 15 than controls. Also, nestlings from carotenoid-supplemented males were in significantly better condition than nestlings of placebo-fed males (Losdat et al. 2010). Thus, our carotenoid supplementation increased the capacity of males to maintain prime body mass and high levels of nestling feeding. One potential explanation for this could be that although carotenoids may be minor antioxidants, the positive effect on body mass may still arise through a direct or indirect contribution to the antioxidant system (Hõrak et al. 2006, 2007; Pérez-Rodríguez 2009). Males may have used supplemental carotenoids to directly or indirectly improve their antioxidant defenses, and then used this additional capacity to increase their metabolic rate and parental contribution while losing less body mass and keeping their oxidative balance undisturbed. Their antioxidant capacity would thus appear unchanged by the carotenoid supplementation.

Alternatively, males may have allocated supplementary carotenoids to other physiological functions. In addition to their potential role as antioxidants, carotenoids, and particularly β-carotene have also been shown to up- and downregulate the immune system (Bendich 1989; Koutsos et al. 2003; Chew and Park 2004; Hartley and Kennedy 2004; Fitze et al. 2007; Costantini and Møller 2009).

Contrary to previous studies on great tits (Isaksson et al. 2007; Isaksson and Andersson 2008), we found a positive correlation between yellow breast coloration and antioxidant capacity 15 days posthatch, that is, toward the end of the stressful and energetically demanding chick-rearing period

Table 4

Linear model testing for an effect of brood size manipulation and carotenoid supplementation on male antioxidant capacity on Day 15 posthatch

Effect	Estimate ± standard error	F_{df}	P
Intercept	1.52 ± 0.46	—	—
Carotenoid treatment[a]	0.13 ± 0.11	$1.54_{1,25}$	0.23
Brood size manipulation[b]	**−1.05± 0.54**	**$2.13_{1,27}$**	**0.16**
Haematocrit	0.05 ± 0.14	$0.13_{1,26}$	0.73
Initial brood size	**−0.05± 0.05**	**$0.55_{1,27}$**	**0.46**
Breast color	**0.09± 0.03**	**$8.25_{1,27}$**	**0.008**
Brood size manipulation* × *initial brood size[b,c]	***0.11* ± *0.07***	***$2.79_{1,27}$***	***0.106***
Initial brood size × breast color	0.00 ± 0.00	$0.03_{1,22}$	0.95
Carotenoid treatment × brood size manipulation[d]	0.17 ± 0.22	$0.55_{1,24}$	0.46
Carotenoid treatment × initial brood size[a]	−0.01±0.07	$0.04_{1,23}$	0.85
Carotenoid treatment × breast color[a]	0.04 ± 0.09	$0.15_{1,21}$	0.70

The model was reduced using a backward stepwise procedure based on AIC (AIC initial = 79.38; AIC final = 70.47). Terms retained in the final model are highlighted in bold. F and P values of terms not retained in the final model are those just before removal.

[a] Relative to the placebo group.

[b] Relative to the control brood group.

[c] Significance of the effect of the breast color if removing this interaction: $F_{1, 28} = 6.51$, $P = 0.016$, AIC = 71.62; if considering the most reduced model: $F_{1, 30} = 4.94$, $P = 0.03$, AIC = 71.02.

[d] Relative to the placebo group and the control brood group.

(Perrins 1965). This is a first indication that this carotenoid-based ornament may mirror male ability to resist oxidative stress (von Schantz et al. 1999). Such a result has important implications for mate choice because females pairing with more colorful males would accrue direct and/or indirect benefits if higher resistance to oxidative stress enables greater parental investment by males (Pike, Blount, Bjerkeng, et al. 2007) and/or if resistance to oxidative stress is heritable (Kim et al. 2010) and offspring sired by colorful males enjoy higher resistance to oxidative stress. The fact that such correlation between color and antioxidant capacity was found only toward the end of the rearing period and after 3 trapping occasions suggests that breast coloration signals a male's ability to maintain antioxidant capacity under particularly stressful circumstances. The correlation between resistance to oxidative stress and coloration (Inouye et al. 2001; Bitton and Dawson 2008; Galván and Møller 2009) could be confounded by age. We did not assess male age and whether resistance to oxidative stress and plumage coloration concomitantly increase with age remains to be tested.

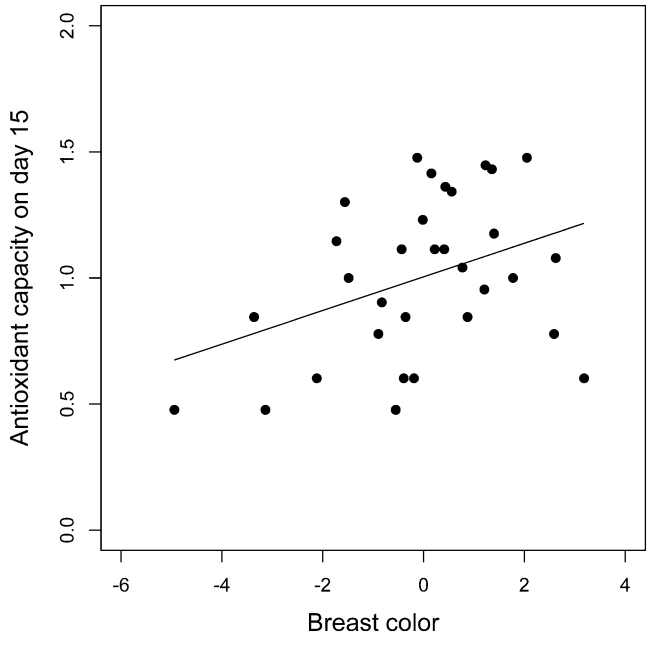

Figure 3
Log-transformed whole blood resistance to a controlled free radical attack (minutes) on Day 15 posthatch in relation to the yellow breast coloration. The line is the linear regression line.

Since carotenoid-based ornaments have been hypothesized to reflect an individual ability to absorb, metabolize, and use carotenoids (Hõrak 2000; Møller et al. 2000; Blount et al. 2003; Koutsos et al. 2003), the most colorful males may be expected to benefit more from carotenoid supplementation. However, we did not find such an effect, which suggests that instead of reflecting antioxidant capacity of carotenoids themselves, carotenoid-based coloration of great tits may reflect plasmatic concentrations of other colorless antioxidants (i.e., vitamins A, C, E, and uric acid; Hartley and Kennedy 2004).

To conclude, our study on a natural free-ranging population of great tits suggests that reproductive effort increases susceptibility to oxidative stress and concurs with previous laboratory studies in identifying oxidative stress as a proximate cost of reproduction. Contrary to previous studies, we also found that yellow breast coloration may signal some male component of resistance to oxidative stress. This may have important consequences for mate choice as females may benefit from mating with colorful males with better resistance to oxidative stress.

FUNDING

Swiss National Science Foundation (3100A0-122566) to HR.

Authors' contributions: designed the study: SL, FH, HR; collected and analyzed the data: SL, BG, FH; wrote the paper: SL, FH, HR; supervised the study: FH, HR. The authors thank DSM Nutritional Products Ltd for generously providing carotenoids, and J. D. Blount, A.P. Møller for helpful comments on the manuscript. This work was conducted under license of the Ethical Committee of the Agricultural Office of the Canton Bern. Ringing permits were provided by the Swiss Federal Agency for Environment, Forests and Landscapes.

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