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LETTER

Sperm of colourful males are better protected against oxidative stress

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Abstract

Sperm cells are highly vulnerable to free radicals, and sperm quality and male fertility are critically affected by oxidative stress. Recently, sexual ornaments, particularly carotenoid-based colourful traits, have been proposed to depend on a male's capacity to resist oxidative stress, and thus to signal sperm quality. We conducted an experimental test of this hypothesis on great tits *Parus major*, in which adults are sexually dichromatic in carotenoid-based breast plumage. We report the first evidence that ornaments and sperm quality may be linked through oxidative stress. When experimentally subjected to oxidative stress resulting from increased workload, less colourful males suffered a greater reduction in sperm motility and swimming ability, and increased levels of sperm lipid peroxidation compared to more colourful males. Moreover, the level of sperm lipid peroxidation was negatively correlated with sperm quality. Finally, carotenoid supplementation increased sperm quality of less colourful males, suggesting that pale males are deficient in carotenoid antioxidants.

Keywords

Carotenoid-based colour, carotenoids, lipid peroxidation, oxidative stress, *Parus major*, sperm motility, sperm quality, sperm velocity.

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INTRODUCTION

Vertebrate spermatozoa are very susceptible to reactive oxygen species (ROS) due to the high proportion of polyunsaturated fatty acids (PUFAs) in their membrane, and their condensed DNA and reduced transcription machinery, which limits DNA repair (Tremellen 2008). Oxidative stress (the imbalance between pro-oxidants and antioxidants in favour of the former) prevails in an animal's life because it can result as a by-product of normal metabolism or from physiological processes involved in immunity or reproduction (Costantini 2008; Monaghan *et al.* 2009). Damage to lipids, proteins and DNA of spermatozoa resulting from oxidative stress are known to reduce fertility in domestic animals and are thought to be a major cause of male infertility in humans (Aitken 1999; Tremellen 2008).

The expression of sexual ornaments has been proposed to reflect past and present exposure to oxidative stress, and to depend on a male's ability to fight free radicals (von Schantz *et al.* 1999). Therefore, it has been hypothesized that sexual ornaments such as carotenoid-based colours should positively reflect sperm quality (Blount *et al.* 2001; Velando *et al.* 2008). Correlational studies showing that male coloration positively correlates with sperm quality (Peters *et al.* 2004; Locatello *et al.* 2006; Pitcher *et al.* 2007) provide a first line of evidence. However, experimental data in support of the hypothesis that sexually selected traits signal sperm quality through the male's ability to protect his sperm from oxidative stress are still lacking. Here, we report on an experimental test of this hypothesis using free-ranging great tit *Parus major* as a model species because they exhibit sexually dichromatic carotenoid-based yellow breast plumage (Partali *et al.* 1987; Delhey & Peters 2008). Carotenoid-based colourful ornaments may advertise their bearer's ability to resist oxidative stress (von Schantz *et al.* 1999; Bertrand *et al.* 2006a; Pike *et al.* 2007) through at least two non-mutually exclusive mechanisms. Carotenoid-based colours may reflect the capacity of a male to cope with the trade-off between carotenoids used

either as antioxidants or as pigments (von Schantz *et al.* 1999), or they may reflect a male's ability to acquire, absorb and metabolize carotenoids (Hill 1992; Møller *et al.* 2000) as well as other more potent, colourless dietary antioxidants, which may in turn protect carotenoid pigments from oxidation and make them accessible to signalling (Hartley & Kennedy 2004; Bertrand *et al.* 2006b).

To increase workload and thereby subject males to oxidative stress, we experimentally increased brood size. Artificial enlargement of the brood is known to force parent birds to increase their feeding effort and to generate oxidative stress (Alonso-Alvarez *et al.* 2004). Five days later, males were captured and a sperm sample collected to investigate how sperm quality was affected by oxidative stress. On this occasion, we also supplemented half of the males with dietary carotenoids while the other half received a placebo treatment. Sperm quality was assessed a second time after 8 days of carotenoid supplementation to assess whether dietary carotenoids modulate the impact of oxidative stress on sperm quality.

MATERIAL AND METHODS

Study organism and brood size manipulation

This study was conducted from 9 May to 23 June 2008 on a great tit population breeding in the forest Forst, Bern, Switzerland. Great tit males exhibit a carotenoid-based yellow breast plumage. Carotenoid-based ornaments have been shown to be sexually selected in a number of species (e.g. in fish and birds; Pitcher *et al.* 2003; Hill 2006). Although no direct evidence exists, a number of studies suggest that carotenoid-based plumage of great tits is likely under sexual selection. It is known to differ greatly between the sexes, to be condition-dependent, to exhibit large between-individual variance and to be related to individual quality (reviewed in Delhey & Peters 2008). In addition, pair members are mated assortatively according to the colour of their breast, which may be a first indication of mutual mate choice based on this ornament (Hegyi *et al.* 2007). Female great tits lay up to 12 eggs, of which *c.* 8% on average fail to hatch (Spottiswoode & Møller 2004). Hatching failure is a common phenomenon in birds that may be caused by male infertility (Birkhead *et al.* 2008). Females also engage in extrapair copulations leading to extrapair fertilizations, and sperm competition can be significant (Lubjuhn *et al.* 1999).

To increase workload and thereby subject males to oxidative stress, we experimentally enlarged 29 broods by adding two nestlings 2 days after hatching. Artificial enlargement of the brood is known to force parent birds to increase their feeding effort, including in the great tit where both parents feed the nestlings and respond to brood size manipulation (Smith *et al.* 1988; Sanz & Tinbergen 1999; Neuenschwander *et al.* 2003). Such an experimental increase in brood size has been shown to induce oxidative stress in captive zebra finch *Taenopygia guttata* (Alonso-Alvarez *et al.* 2004). In the present experiment, we found that our brood size manipulation indeed impaired male antioxidant capacity, which is a first piece of evidence that males underwent oxidative stress (Losdat, S., Helfenstein, F., Gaude, B. & Richner, H., unpublished data). As a control group, we visited another 31 nestboxes, but left brood size unchanged. Males did not differ between groups in initial brood size, laying date, tarsus length and wing length (all $|t| < 1.46$, $P > 0.16$, $n = 56$). Nestling mortality between Day 2 and Day 15 was unaffected by our treatments (generalized linear model with binomial error distribution and logit link function, deviance scaled to unity to correct for overdispersion; brood size manipulation: $F_{1,52} = 1.23$, $P = 0.27$; carotenoid supplementation: $F_{1,52} = 2.09$, $P = 0.15$; Brood size manipulation × Carotenoid supplementation: $F_{1,52} = 1.37$, $P = 0.25$), and male breast coloration was unrelated to initial brood size ($F_{1,54} = 0.26$, $P = 0.62$). Males with enlarged broods tended to be more colourful ($t < 1.94$, $P = 0.06$, $n = 56$). However, this tendency should not confound our results as it is expected to oppose the negative effect of brood enlargement, thus rendering our analyses more conservative. In addition, breast colouration is a variable of interest that is hypothesized to predict male ability to resist oxidative stress. For this reason, breast colour is included as an explanatory variable in all analyses, thus correcting for any potential confounding effect.

Carotenoid supplementation

On Day 7 post-hatch, males were randomly assigned to a carotenoid supplementation or a placebo treatment by force-feeding them either with one *Calliphora* spp. larva coated with a mixture of corn oil, lutein, zeaxanthin and β-β carotene (carotenoid-supplemented), or one larva coated with corn oil only (placebo). Carotenoids were provided in the relative proportions found in natural great tit food (80% lutein, 3% zeaxanthin – the two pigments found in great tit feathers – and 17% β-β carotene; Partali *et al.* 1987). Males were captured again on Day 11 and the carotenoid supplementation was repeated. On each occasion we provided four times the daily amount of carotenoids that males obtain from their natural diet (Helfenstein *et al.* 2008). Carotenoids are lipid-soluble antioxidants that birds can store in their liver (Surai 2002) and our mode of supplementation aimed at doubling, on average, the daily intake of carotenoids over the entire experimental period. Of the males that comprise our final sample, three could not be re-captured on Day 11. This small number prevents us from accounting for this variation statistically. However, these non-recaptured males render our analyses conservative as they received a single dose of carotenoids instead of two.

Our experiment appeared very stressful to the birds, and we were unable to collect blood samples large enough to extract data about plasmatic carotenoid concentrations. Thus, we could not check the efficacy of our supplementation method. However, we supplemented small, physiological doses of carotenoids, and we would not have expected supplemented males to show elevated levels of plasma carotenoids. Birds can store the lipid-soluble carotenoids in their liver so that levels of circulating carotenoids may remain unchanged. Additionally, small amounts of supplemental carotenoids, a limiting resource animals can only acquire from their food, are likely used immediately by wild, free-ranging birds.

Of the 60 males captured on Day 7, 26 males could not be recaptured on Day 15, but the probability to recapture a male on Day 15 was independent of the treatments he received (brood size manipulation: $\chi^2 = 1.80$, $P = 0.18$; carotenoid supplementation: $\chi^2 = 0.09$, $P = 0.77$). These males also did not differ from recaptured males in terms of laying date, initial brood size, body mass, tarsus length or wing length (all $|t| < 1.75$, $P > 0.09$, $n = 59$–60). However, males that were not recaptured tended to be paler (breast plumage coloration: $t = 1.87$, $P = 0.07$, $n = 60$). Nevertheless, this tendency should not confound our results as non-recaptured birds are randomly distributed among groups. Sperm quality on Day 7 did not differ significantly depending on whether males were recaptured on Day 15 or not (percentage of motile sperm: $F_{1,54} < 0.01$, $P = 0.97$; sperm swimming ability: $F_{1,54} < 0.01$, $P = 0.98$). Finally, males recaptured on Day 15 did not differ among groups in terms of laying date, initial brood size, wing length and breast colour (all $F_{1,30} < 2.75$, $P > 0.11$), although non-supplemented males caring for unmanipulated broods tended to have smaller tarsi ($F_{1,30} = 3.84$, $P = 0.06$). If smaller males are poorer males, this may produce a spurious positive effect of the carotenoid supplementation. However, including tarsus length in our analyses to correct for such a bias does not qualitatively change our results.

Breast plumage colouration

We recorded reflectance spectra of the males' yellow breast plumage according to standard procedures (Andersson & Prager 2006) with a spectrophotometer, a bifurcated reflectance probe and a UV/visible/near-infrared light source (Ocean Optics Inc., Duiven, The Netherlands). Colour vision in birds depends on four types of single cones that are sensitive to very short (VS), short (S), medium (M) and long (L) wavelengths (Kelber *et al.* 2003). We used physiological models of colour vision (Vorobyev *et al.* 1998) to describe breast colouration as perceived by conspecifics. Four cone quantum catches (Vorobyev *et al.* 1998) were calculated, transformed and projected into a three-dimensional Euclidean space (Kelber *et al.* 2003). Male breast colour was then characterized using PC1 of a principal component analysis of the x, y and z Euclidean coordinates (Peters *et al.* 2008). Along this axis, colourful males with yellower plumage have positive scores while paler males have negative scores (see Appendix S1 for more details). All measures were performed blindly with respect to treatments.

Sperm quality analyses

Ejaculates (*c.* 0.5 μL) were collected by gently massaging the male's cloaca (Wolfson 1952) on Days 7 ($n = 56$; we failed to collected sperm of four males) and 15 post-hatch ($n = 34$). Our study took place at the nestling stage, when males can be easily trapped while feeding their progeny. We believe that studying sperm quality at this stage is biologically relevant for the following reasons. First, great tits can produce replacement and/or second clutches, and sperm quality remains critical for males to secure their paternity in these broods. Second, the breeding season stretches over several weeks so that some pairs may be in the laying phase when others are at the nestling stage. Great tit females engage in extrapair copulations and sperm competition is significant (Lubjuhn *et al.* 1999). Sperm quality is thus crucial for males to gain fitness through extrapair fertilizations throughout the whole breeding season.

Sperm were immediately mixed with 50 μL of prewarmed Dulbecco's modified Eagle's medium and 5 μL of sperm/buffer solution was immediately transferred under a microscope with dark-field condition and sperm motion was video-recorded for 3 min at 40 °C. Sperm motion was analysed after 0, 60, 120 and 180 s of recording using a CASA plug-in to ImageJ (Wilson-Leedy & Ingermann 2007). We recorded the percentage of motile sperm and mean values for VCL (curvilinear velocity, total distance travelled, $\mu m\ s^{-1}$), VAP (average path velocity, smoothed path using roaming average, $\mu m\ s^{-1}$), VSL (straight line velocity, distance from origin to end point, $\mu m\ s^{-1}$), straightness (VSL/VAP, path curvature), wobble (VAP/VCL, side to side movement of the sperm head, also described as the oscillation of the actual trajectory about its average path), BCF (beat cross frequency, the frequency at which VCL crosses VAP, Hz), progression (PROG: average distance from origin on the average path during all frames analysed) and efficiency (PROG/VAP: portion of generalized motion resulting in movement away from the origin). Sperm motility and sperm swimming velocity are determinant components of male fertility and sperm competitive ability (Pizzari & Parker 2009). Hence, sperm quality was assessed as (1) percentage of motile sperm and (2) PC1 scores from a principal component analysis of the other eight variables plus the number of sperm cells detected by the CASA software. This axis describes 'sperm

swimming ability', i.e. sperm swimming fast, straightforwardly and efficiently (fewer overall movements to achieve greater progression; see Appendix S2 for more details). All measures were performed blindly with respect to treatments.

Sperm lipid peroxidation

When possible, a second ejaculate (0.5–1.5 μL) was collected (Day 7: $n = 45$; Day 15: $n = 34$) with a graduated 5 μL capillary tube, transferred into 10 μL PBS and frozen at −80 °C until analysed. Malondialdehyde (MDA) concentration, a marker of lipid peroxidation (Monaghan *et al.* 2009), was assessed using HPLC and a standard curve established by serial dilutions of 1,1,3,3-tetraethoxypropane (TEP) (Mougeot *et al.* 2009; see Appendix S3 for more details). Analyses were carried out blindly with respect to treatments.

Predictions

From our experimental design we derived the following predictions. Increasing brood size induces oxidative stress (Alonso-Alvarez *et al.* 2004), and brood size manipulation is predicted to negatively affect sperm quality and lead to increased levels of lipid peroxidation in ejaculates. Brood size was increased by way of two additional nestlings and this effect is predicted to be more pronounced in initially smaller broods. The oxidation-dependent hypothesis (Blount *et al.* 2001; Velando *et al.* 2008) predicts that more colourful males are better able to protect their sperm against the deleterious effect of oxidative stress. This hypothesis leads to the prediction that, among males with enlarged broods, paler males with initially small broods are expected to suffer more from brood enlargement and subsequent oxidative stress.

Concerning the carotenoid supplementation, we derived the following predictions. Although debated, carotenoids are hypothesized to function as antioxidants particularly in situations of acute oxidative stress (Costantini & Møller 2008). We thus predicted an interaction between brood size manipulation and carotenoid supplementation, with males caring for enlarged broods being expected to benefit more from a carotenoid supplementation. Carotenoid-based colours have been hypothesized to reflect a male's ability to assimilate and use carotenoids (Hill 2006), According to this hypothesis, colourful males are predicted to benefit more from a carotenoid supplementation.

Statistical analyses

Both the percentage of motile sperm and sperm swimming ability showed a marked decline with time (generalized linear mixed model for repeated measures, REML-GLMM, with bird identity as the random subject and time as the repeat; percentage of motile sperm: $\beta \pm SE = -0.002 \pm 0.0005$, $F_{1,59} = 16.12$, $P = 0.0002$; sperm swimming ability: $\beta \pm SE = -0.0008 \pm 0.00007$, $F_{1,59} = 136.3$, $P < 0.0001$), as is commonly reported in studies on sperm motility and velocity (Holt *et al.* 1997; Froman & Feltmann 2000; Levitan 2000). We therefore chose to average values per individual and conducted more simple GLMs. These averages partly include a time effect as two individuals with similar initial values of sperm swimming ability or percentage of motile sperm but producing sperm having different temporal dynamics will have different mean values. Nevertheless, more complicated analyses based on REML-GLMMs for repeated measures yielded qualitatively similar results. Therefore, we only report results from GLMs. To account for potential seasonal variation in sperm quality, we included the date of first capture in all models. Due to the numerous problems entailed in stepwise model-reduction methods (Whittingham *et al.* 2006), we based inference on full models with all predictors present. However, model selection based on maximum-likelihood estimation procedures and AIC always led to the same conclusions.

RESULTS

Effect of increased workload

On Day 7 post-hatch, i.e. after 5 days of increased workload, the percentage of motile sperm was found to significantly depend on an interaction between brood enlargement, initial brood size and breast plumage colour (Table 1; Fig. 1). Males caring for initially small broods and exhibiting paler breast plumage suffered more from brood enlargement and resulting oxidative stress (Fig. 1b). We found similar results for sperm swimming ability (Table 1).

Both the percentage of motile sperm and sperm swimming ability were found to correlate negatively with ejaculate concentrations of MDA, a reliable marker of lipid peroxidation (percentage of motile sperm: intercept: 0.66 ± 0.08, MDA concentration: $\beta = -0.082 \pm 0.039$, $F_{1,39} = 4.36$, $P = 0.04$; sperm swimming ability: intercept: 1.06 ± 0.42, MDA concentration: $\beta = -0.45 \pm 0.20$, $F_{1,39} = 5.20$, $P = 0.03$; Fig. 2). We found a marginally non-significant trend for ejaculate MDA concentration to depend on brood enlargement, initial brood size and breast plumage colour (three-way interaction; see below): when subjected to oxidative stress, paler males with smaller initial broods tended to have greater MDA concentration in their ejaculate (intercept: 2.89 ± 2.51; date of capture: -0.02 ± 0.02, $F_{1,36} = 0.72$, $P = 0.40$; brood enlargement: $\beta = 1.01 \pm 1.10$, $F_{1,36} = 0.84$, $P = 0.37$; initial brood size: $\beta = 0.10 \pm 0.11$, $F_{1,36} = 0.20$, $P = 0.66$; breast colour: $\beta = 0.37 \pm 0.68$, $F_{1,36} = 2.64$, $P = 0.11$; Brood enlargement ×

Table 1 GLM investigating the impact of brood size manipulation (unmanipulated control broods vs. enlarged broods) on the percentage of motile sperm (square-root transformed) and sperm swimming ability in ejaculates collected 7 days post-hatch, i.e. after 5 days of increased workload

Effect	Estimate ± SE	$F_{d.f.}$	P
Percentage of motile sperm			
Intercept	−0.23 ± 0.54	–	–
Date of capture	0.007 ± 0.005	$1.93_{1,47}$	0.17
Brood size manipulation*	−0.45 ± 0.25	$3.18_{1,47}$	0.08
Initial brood size	0.004 ± 0.023	$4.10_{1,47}$	0.05
Breast colour	−0.05 ± 0.19	$5.25_{1,47}$	0.03
Brood size manipulation × Initial brood size*	0.05 ± 0.03	$2.70_{1,47}$	0.11
Brood size manipulation × Breast colour*	0.72 ± 0.26	$7.74_{1,47}$	0.008
Initial brood size × Breast colour	0.013 ± 0.031	$4.43_{1,47}$	0.04
Brood size manipulation × Initial brood size × Breast colour*	−0.091 ± 0.031	$8.45_{1,47}$	0.0055
Sperm swimming ability			
Intercept	−9.00 ± 2.89	–	–
Date of capture	0.09 ± 0.026	$11.97_{1,47}$	0.001
Brood size manipulation*	−1.09 ± 1.34	$0.65_{1,47}$	0.42
Initial brood size	0.13 ± 0.12	$6.56_{1,47}$	0.01
Breast colour	−1.40 ± 0.94	$0.01_{1,47}$	0.97
Brood size manipulation × Initial brood size*	0.13 ± 0.17	$0.65_{1,47}$	0.42
Brood size manipulation × Breast colour*	2.85 ± 1.38	$4.25_{1,47}$	0.05
Initial brood size × Breast colour	0.18 ± 0.11	$0.01_{1,47}$	0.97
Brood size manipulation × Initial brood size × Breast colour*	−0.35 ± 0.17	$4.46_{1,47}$	0.04

*Enlarged brood group relative to unmanipulated control group.

Initial brood size: $\beta = -0.13 \pm 0.13$, $F_{1,36} = 0.97$, $P = 0.33$; Brood enlargement × Breast colour: $\beta = -3.43 \pm 1.65$, $F_{1,36} = 4.32$, $P = 0.045$; Initial brood size × Breast colour: $\beta = -0.04 \pm 0.08$, $F_{1,36} = 2.30$, $P = 0.14$; Brood enlargement × Initial brood size × Breast colour: $\beta = 0.39 \pm 0.20$, $F_{1,36} = 3.89$, $P = 0.056$).

Effect of carotenoid supplementation

After 13 days of increased workload and 8 days of carotenoid supplementation (Day 15 post-hatch), the percentage of motile sperm was found to be significantly influenced by (1) an interaction between brood enlargement treatment and carotenoid supplementation, with males caring for enlarged broods benefiting from carotenoid supplementation (Fig. 3), (2) an interaction between carotenoid supplementation and breast plumage colour, with males exhibiting a paler breast benefiting more from carotenoid supplementation (Fig. 4a) and (3) an interaction between carotenoid supplementation and initial brood size, with males caring for initially larger broods benefiting more from carotenoid supplementation (Fig. 4b; Table 2). However, sperm swimming ability was found to be unaffected by our treatments (all terms $F_{1,22} < 1.84$, $P > 0.21$).

On Day 15, neither the percentage of motile sperm nor sperm swimming ability were significantly correlated with ejaculate MDA concentration ($F_{1,27} = 1.00$, $P = 0.32$; $F_{1,27} = 0.82$, $P = 0.37$). Ejaculate MDA concentration was also not significantly affected by our treatments, although males caring for large broods showed higher concentrations of MDA in their ejaculates ($\beta = 0.13 \pm 0.12$, $F_{1,22} = 5.54$, $P = 0.03$; date of capture: $\beta = 0.04 \pm 0.02$, $F_{1,22} = 4.58$, $P = 0.04$; all other terms $F_{1,22} < 2.96$, $P > 0.10$).

DISCUSSION

After 5 days of increased workload, males with paler breast plumage suffered more from elevated oxidative stress (brought about by a 50% increase in brood size) and produced ejaculates with a lower percentage of motile sperm and spermatozoa with reduced swimming ability. In addition, both the percentage of motile sperm and sperm swimming ability were found to correlate negatively with ejaculate concentrations of MDA, a reliable marker of lipid peroxidation. Finally, paler males caring for initially small broods tended to have higher levels of MDA in their ejaculates, suggesting that the reduction in sperm quality of paler males was at least partially due to higher levels of lipid peroxidation. In species with seasonal reproduction, males frequently face situations of acute oxidative stress when their metabolic rate steeply increases and they spend increasing time and energy in territorial defence, mate guarding and other mating and breeding activities (Nilsson 2002). Oxidative stress is indeed hypothesized to be the

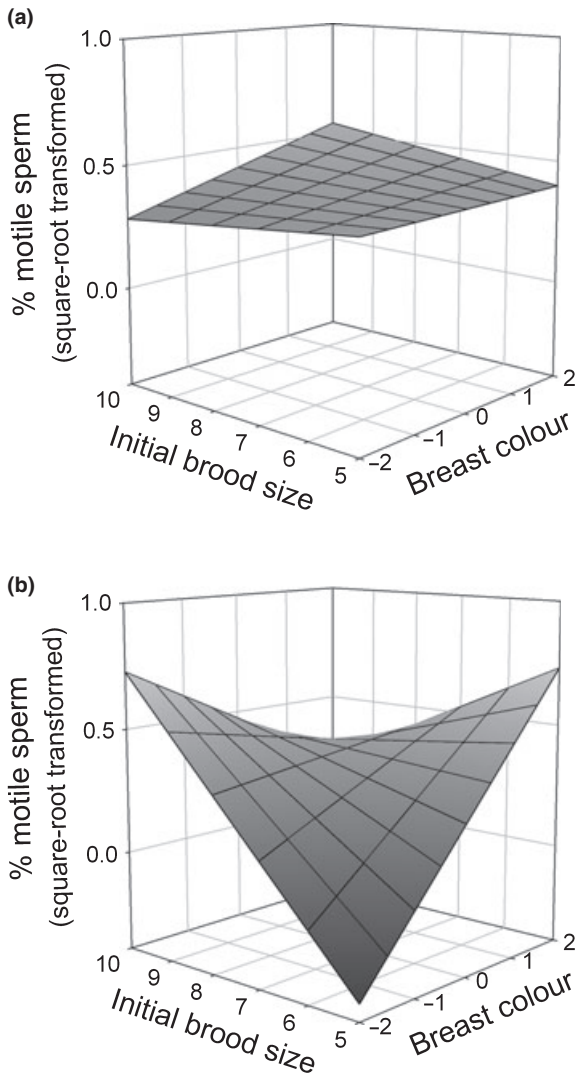

Figure 1 Variation in the percentage of motile sperm in relation to the brood size manipulation, male's breast plumage colour, initial brood size. (a) Control group. (b) Enlarged brood group. Graphics were built using the predicted values derived from the model (explanatory variables within their 95% range).

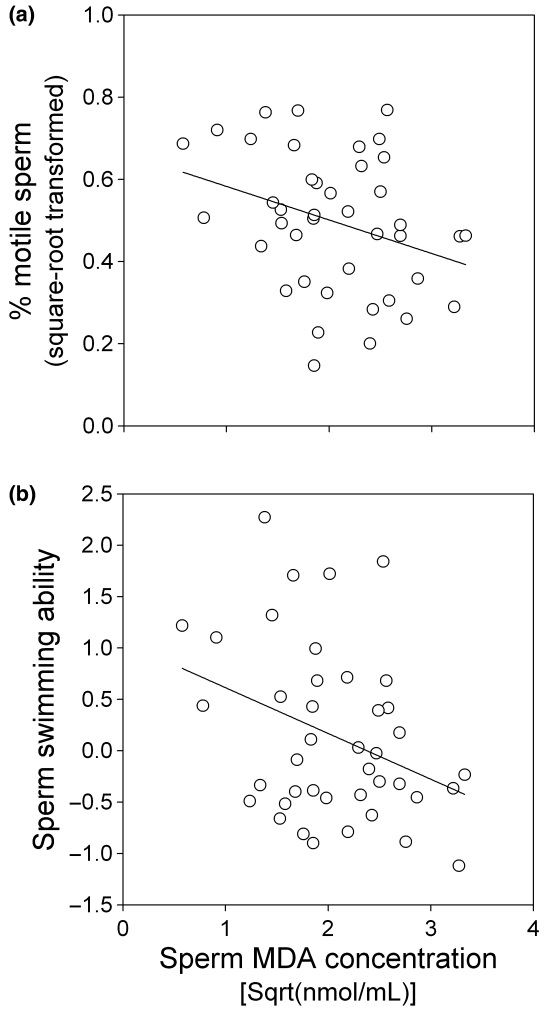

Figure 2 Percentage of motile sperm (a) and sperm swimming ability (b) in relation to sperm malondialdehyde (MDA) concentration. The lines are the linear regression lines.

major proximate cost of reproduction (Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004).

On Day 15, i.e. after 13 days of oxidative stress, the percentage of motile sperm was found to be only weakly affected (Fig. 3; non-significant differences between non-supplemented males caring for increased broods and males caring for unchanged broods; Scheffe *post hoc* tests, $P > 0.21$), and sperm swimming ability and sperm MDA concentration were unaffected, by brood size manipulation. This suggests that, during the course of our experiment, males have developed compensatory antioxidant mechanisms, such as enhanced antioxidant enzyme synthesis (Monaghan *et al.* 2009), which limited the negative impact of brood enlargement on sperm quality.

On Day 15, i.e. after 8 days of carotenoid supplementation, we found the percentage of motile sperm, but not sperm swimming ability, to be improved by carotenoid supplementation in males that cared for enlarged broods (Fig. 3). These results suggest a moderate action of carotenoids, and they are in line with previous studies showing that carotenoids are secondary antioxidants acting only in situations of acute oxidative stress (Costantini & Møller 2008). Carotenoids can be found in small quantities in avian semen (Rowe & McGraw 2008), and a direct antioxidant protection of carotenoids to sperm therefore seems possible. Alternatively, supplementary carotenoids may have indirectly enhanced overall organism-level antioxidant capacity, enabling better protection of spermatozoa

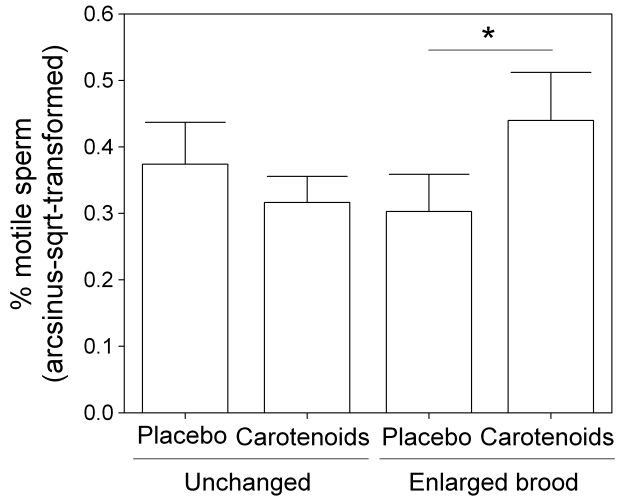

Figure 3 Percentage of motile sperm in relation to brood enlargement and carotenoid supplementation (means ± SE). Among males subjected to oxidative stress (enlarged brood), carotenoid-supplemented males produced sperm of greater motility than males that received a placebo (Scheffe *post hoc* test: $P = 0.038$, indicated by an asterisk in the figure).

against ROS. Moreover, carotenoids can recycle vitamin E, which itself is probably the major compound responsible for antioxidant protection in sperm phospholipids (Surai 2002; Monaghan *et al.* 2009).

Among males that received a placebo dietary treatment, paler males produced less motile sperm than brightly coloured males (Fig. 4a dashed regression line), whereas paler males that were carotenoid-supplemented achieved values of sperm motility similar to colourful males (Fig. 4a, continuous regression line), suggesting that paler males were deficient in dietary antioxidants. Finally, we found an interaction between carotenoid supplementation and initial brood size (Fig. 4b), with carotenoid-supplemented males caring for larger initial broods producing more motile sperm, suggesting that males of higher phenotypic quality, i.e. producing larger broods (Pettifor *et al.* 2001), are better able to assimilate carotenoids.

Our brood size manipulation indirectly generated oxidative stress and our results may be partly due to males with enlarged broods having re-allocated their energy from spermatogenesis to some other functions related to parental care. However, the increase in MDA concentration in ejaculates of males caring for enlarged broods, the positive effect of carotenoid supplementation on sperm quality in males caring for enlarged broods and the positive effect of carotenoid supplementation on sperm quality in paler males clearly support a role for oxidative stress in the deterioration of sperm quality.

Our study provides the first evidence that sexual ornaments may reliably signal sperm quality through the

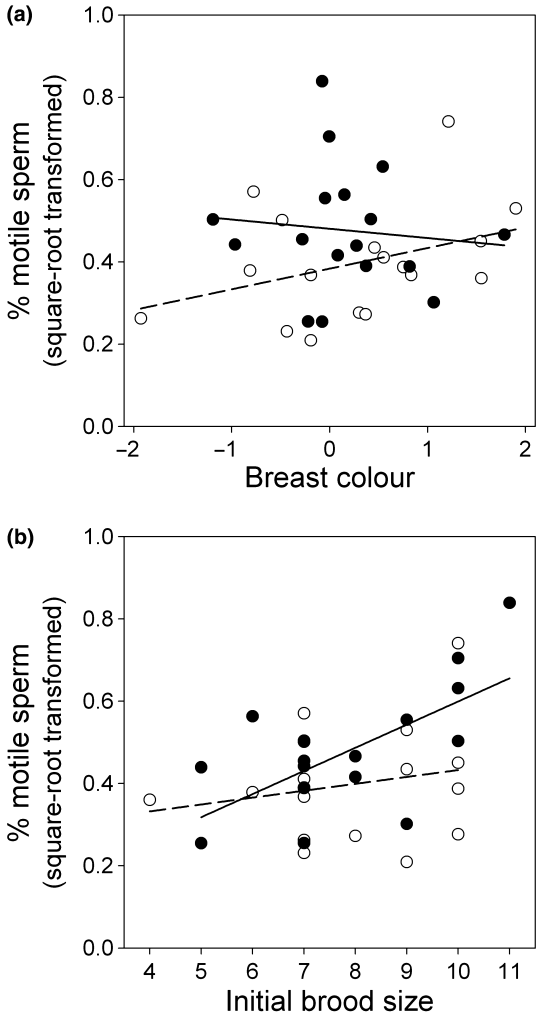

Figure 4 Percentage of motile sperm in relation to carotenoid supplementation, breast colour and initial brood size. (a) Among males that received a placebo, pale males produced less motile sperm (open circles and dashed regression line) than colourful males, whereas when they received additional carotenoids pale males produced sperm that were as motile as those of colourful males (dots and solid regression line). (b) There was no relationship between initial brood size and sperm motility among males that received a placebo (open circles and dashed regression line), whereas males caring for larger initial broods benefited more from additional carotenoids (dots and solid regression line). Results remained qualitatively unchanged when we removed the male with the most negative breast colour value or the males with initial broods of 4 and 11.

male's capacity to protect his sperm from oxidative stress. Sperm swimming ability and sperm motility are determinant components of male fertility and sperm competitive ability (Pizzari & Parker 2009). Our results have important evolutionary implications because variations in the ability of males to protect their sperm from oxidative stress may explain variations in sperm senescence rate (Pizzari *et al.*

Table 2 GLM investigating the impact of brood size manipulation (unmanipulated control broods vs. enlarged broods) and carotenoid supplementation on the percentage of motile sperm (square-root transformed) in ejaculates collected 15 days post-hatch, i.e. after 13 days of increased workload and 8 days of carotenoid supplementation

Effect	Estimate ± SE	$F_{d.f.}$	P
Intercept	−0.03 ± 0.46	–	–
Date of capture	0.008 ± 0.004	$3.62_{1,22}$	0.07
Brood size manipulation*	0.06 ± 0.28	$0.45_{1,22}$	0.51
Carotenoid supplementation†	−1.13 ± 0.26	$15.64_{1,22}$	0.0007
Initial brood size	−0.05 ± 0.03	$0.57_{1,22}$	0.46
Breast colour	−0.24 ± 0.19	$3.42_{1,22}$	0.08
Brood size manipulation × Carotenoid supplementation‡	0.25 ± 0.08	$10.24_{1,22}$	0.004
Brood size manipulation × Initial brood size*	−0.015 ± 0.032	$0.21_{1,22}$	0.65
Brood size manipulation × Breast colour*	−0.09 ± 0.05	$3.26_{1,22}$	0.08
Carotenoid supplementation × Initial brood size†	0.14 ± 0.03	$17.13_{1,22}$	0.0004
Carotenoid supplementation × Breast colour†	−0.13 ± 0.05	$5.92_{1,22}$	0.02
Initial brood size × Breast colour	0.05 ± 0.02	$4.00_{1,22}$	0.06

*Enlarged brood group relative to unmanipulated control group.
†Carotenoid-supplemented group relative to placebo group.
‡Enlarged brood, carotenoid-supplemented group relative to all other groups.

2008; White *et al.* 2008; Møller *et al.* 2009) and in the outcome of sperm competition (Pizzari *et al.* 2008; White *et al.* 2008).

These results also illustrate a potential mechanism linking male ornaments to sperm quality (Peters *et al.* 2004; Locatello *et al.* 2006; Pitcher *et al.* 2007), a premise to the phenotype-linked fertility hypothesis (Sheldon 1994). Females choosing to copulate with more ornamented males would gain direct fitness benefits by fertilizing their eggs with less oxidatively damaged sperm, hence avoiding the risk of infertility associated with oxidatively damaged sperm (Tremellen 2008). Additionally, oxidative damages to sperm DNA have been shown to translate into deleterious mutations in the zygote (Tremellen 2008). Females would thus not only avoid infertility but also avoid producing low-quality offspring bearing heritable deleterious mutations.

The good-sperm model proposes that if sperm quality and sperm competitive ability are determined by heritable differences in male quality (i.e. viability), females may be selected to mate multiply because they would gain indirect fitness benefits by producing sons with both higher fertilization success and higher viability (Yasui 1997). Variations in resistance to oxidative stress may underlie variations in survival ability (Monaghan *et al.* 2009). Therefore, if a male's ability to resist oxidative stress is heritable, our results may also offer a mechanism by which sperm quality and viability would be associated thus favouring female promiscuity as proposed by the good-sperm hypothesis (Yasui 1997).

AUTHOR CONTRIBUTION

F.H. and S.L. designed the study, collected and analysed the data. F.H. wrote the paper. A.P.M. and J.D.B. contributed new techniques and analysis tools. H.R. supervised the study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Breast plumage colouration.
Appendix S2 Sperm quality analyses.
Appendix S3 Sperm lipid peroxidation.

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Supporting Information to Helfenstein *et al*. "Sperm of colourful males are better protected against oxidative stress"

Appendix S1 *Breast plumage colouration*

We recorded reflectance spectra of male breast yellow plumage using a USB4000 spectrophotometer, a FCR-7UV200-2-ME bifurcated reflectance probe with a 200 μm fibre core diameter, and a deuterium-halogen/tungsten light source (DH-2000-BAL, UV-VIS-NIR; Ocean Optics Inc., Netherlands). The tip of the probe was fitted with a black PVC cylinder to standardize measuring distance and exclude ambient light (Andersson & Prager 2006). The probe was held perpendicular to the plumage surface (Andersson & Prager 2006). Each measurement was the average of four scans with a 100 ms integration time, and was calculated relative to a diffuse reflectance standard (WS-1, Ocean Optics Inc., The Netherlands). The spectrophotometer was calibrated before each individual was measured. We took two series of reflectance readings on four different patches (on both sides of the keel at the furculum and on both flanks) to assess repeatability. We computed four cone quantum catches (Vorobyev *et al.* 1998) using data on cone spectral sensitivities and ocular media transmittance from the closely related blue tit *Cyanistes caeruleus* (Hart *et al.* 2000), and the forest shade irradiance spectrum (Endler 1993) because our great tit population breeds in forests. After accounting for colour constancy by applying von Kries algorithm (Kelber *et al.* 2003), we transformed relative cone quantum catch to project the tetrahedral avian visual space into a three-dimension Euclidean space (Kelber *et al.* 2003). In this space, 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. Male breast colour was then characterized using PC1 of a

principal component analysis of the x, y and z Euclidean coordinates (Peters *et al.* 2008). PC1 explained 84.7 % of the variance, opposed colourful males with yellower plumage (positive scores) to paler males (negative scores). PC1 scores were repeatable within males across patches and repeats ($r = 0.15$, $P < 0.0001$, $n = 60$) and strongly correlated with carotenoid chroma ($r = 0.74$, $P < 0.0001$, $n = 60$), a spectral measure of the amount of carotenoids deposited in the feathers (Andersson & Prager 2006). All measures were done blindly with respect to treatments and repeated measures were averaged per male.

Appendix S2 *Sperm quality analyses*

We recorded the percentage of motile sperm and mean values for VCL, VAP, VSL, straightness, wobble, BCF, progression and efficiency. We also recorded the number of sperm cells detected by the CASA plug-in because moving sperm are likely to interfere more with each other at higher densities and this may affect sperm mobility parameters. Sperm swimming behaviour has previously been described using various parameters associated with male fertility such as VSL, VAP, VCL or a combination of these parameters (Holt *et al.* 1997; Froman & Feltmann 2000; Soler *et al.* 2003; Gage *et al.* 2004; Pizzari *et al.* 2004; Denk *et al.* 2005). We do not know which of the measured parameters are the most relevant to describe sperm swimming behaviour in the great tit. Moreover, most of these parameters are correlated with each others (sperm samples collected on day 7 post-hatch: |r| range: 0.01 – 0.99; and on day 15 post-hatch: |r| range: 0.37 – 0.99). Therefore, we chose to synthesise these sperm mobility variables, plus the number of sperm cells detected, using principal component analysis (using the varimax approach to the correlation matrix). PC1 for sperm samples collected on day 7 post-hatch explained 66.4 % of the variance and was positively correlated with VSL, VAP, VCL, straightness, wobble, progression and efficiency (all $r > 0.74$; all $P < 0.0001$), and uncorrelated with BCF ($r = -0.12$, $P = 0.37$) and the density of sperm in the sample ($r = 0.02$, $P = 0.89$). PC1 for sperm samples collected on day 15 post-hatch explained 64.1 % of the variance and was positively correlated with VSL, VAP, VCL, straightness, wobble, progression and efficiency (all $r > 0.84$; all $P < 0.0001$), and uncorrelated with BCF ($r = -0.05$, $P = 0.77$) and the density of sperm in the sample ($r = -0.04$, $P = 0.82$). On both dates, the first PC thus describes “sperm swimming ability” i.e. sperm swimming fast, straightforwardly and efficiently (fewer overall movements to achieve greater progression). All measures were done blindly with respect to treatments. Re-analysis of sperm motion at 0

second for all males sampled on day 7 showed significant repeatability of our measures of sperm quality (percentage of motile sperm: r = 0.56, $P < 0.0001$, n = 56; sperm swimming ability: r = 0.57, $P < 0.0001$, n = 56).

Appendix S3 *Sperm lipid peroxidation*

Ejaculate concentrations of malondialdehyde (MDA), formed by the β-scission of peroxidized fatty acids, were assessed using HPLC with fluorescence detection, as described previously (Mougeot *et al.* 2009) with some modifications. All chemicals were HPLC grade, and chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore, Watford, UK). Samples were first immersed in a water bath (ice cold) and sonicated for 10 minutes, then microtubes were homogenized for one minute in a microtube using a motorized pestle, before being centrifuged at 13,000 rpm and 4 °C for 4 minutes. Sample derivitization was done in 2 ml capacity screw-top microcentrifuge tubes. To a 5 µl aliquot of sample or standard (1,1,3,3-tetraethoxypropane, TEP; see below) 5 µl butylated hydroxytoluene solution (0.05% w/v in 95 % ethanol), 40 µl phosphoric acid solution (0.44 M), and 10 µl thiobarbituric acid (TBA) solution (42 mM) were added. Samples were capped, vortex mixed for 5 seconds, then heated at 100°C for exactly 1 hour in a dry bath incubator to allow formation of MDA-TBA adducts. Samples were then cooled on ice for 5 minutes, before 80 µl n-butanol was added and tubes were vortex mixed for 10 seconds. Tubes were then centrifuged at 13,000 rpm and 4 °C for 4 minutes, before a 55 µl aliquot of the epiphase was collected and transferred to an HPLC vial for analysis. Samples (40 µl) were injected into a Dionex HPLC system (Dionex Corporation, California, USA) fitted with a 2 µm pre-column filter and a Hewlett-Packard Hypersil 5µ ODS 100 x 4.6 mm column maintained at 37°C. The mobile phase was methanol-buffer (40:60, v/v), the buffer being a 50mM anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5M potassium hydroxide solution), running isocratically over 3.5 min at a flow rate of 1 $ml.min^{-1}$. Data were collected using a fluorescence detector (RF2000; Dionex) set at 515 nm (excitation) and 553 nm (emission). For calibration a standard curve was prepared using a TEP stock solution (5µM in

40% ethanol) serially diluted using 40% ethanol. TEP standards assayed in triplicate showed high repeatability ($r = 0.99$, $P < 0.0001$, $n = 12$). Analyses were done blindly with respect to treatments.

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