doi:10.1111/j.1420-9101.2008.01591.x

The oxidation handicap hypothesis and the carotenoid allocation trade-off

C. ALONSO-ALVAREZ,* L. PÉREZ-RODRÍGUEZ,*,† R. MATEO,* O. CHASTEL‡ & J. VIÑUELA*

*Instituto de Investigación en Recursos Cinegéticos, IREC (CSIC, UCLM, JCCM), Ciudad Real, Spain
†School of Biological Sciences, University of Aberdeen, Zoology Building, Aberdeen, UK
‡Centre d'Etudes Biologiques de Chizé-CNRS, Villiers en Bois, France

Keywords:

antioxidants;
carotenoids;
coloured traits;
glutathione;
lipid peroxidation;
oxidative stress;
physiological trade-off;
red-legged partridges;
resource allocation;
testosterone.

Abstract

The oxidation handicap hypothesis proposes that testosterone mediates the trade-off between the expression of secondary sexual traits and the fight against free radicals. Coloured traits controlled by testosterone can be produced by carotenoid pigments (yellow–orange–red traits), but carotenoids also help to quench free radicals. Recently, it has been shown that testosterone increases the amount of circulating carotenoids in birds. Here, a testosterone-mediated trade-off in the carotenoid allocation between colour expression and the fight against oxidative stress is proposed. Male red-legged partridges were treated with testosterone, anti-androgens or manipulated as controls. Testosterone-treated males maintained the highest circulating carotenoid levels, but showed the palest red traits and no evidence of oxidative damage. Increased levels of a key intracellular antioxidant (i.e. glutathione) indicated that an oxidative challenge was in fact induced but controlled. The trade-off was apparently solved by reducing redness, allowing increased carotenoid availability, which could have contributed to buffer oxidative stress.

Introduction

In a classical evolutionary trade-off, individuals investing more resources in reproduction would have fewer resources to invest in self-maintenance (Zera & Harshman, 2001). Among the investments necessary for reproduction, the expression of sexual signals is relevant because the stronger their level, the higher the mating chance, and ultimately, the better the reproductive outcome (Maynard Smith & Harper, 2003). Sexual signals are important not only for the bearer, but for the receiver too, which also compromises its own fitness when assessing the trait. Hence, theory predicts that sexual signals are subject to strong selective pressure based on reliability. Only costly traits able to honestly indicate the quality of the bearer should therefore evolve (i.e. the handicap principle; Zahavi, 1975).

Folstad & Karter (1992) refined this principle further proposing the 'immunocompetence handicap hypothesis', which is based on the fact that the expression of many sexual signals is controlled by testosterone in males. Testosterone would act as a 'double-edged sword' by promoting the expression of sexual signals but simultaneously suppressing immune function (Roberts *et al.*, 2004). Thus, only high-quality males would be able to withstand the immunosuppressive effect, assuring the honesty of the signal.

However, the physiological costs associated with testosterone can also be based on oxidative stress, which results from the imbalance between the production of reactive oxygen species (ROS) and the antioxidant defences (Finkel & Holbrook, 2000). ROS are unstable by-products of normal metabolism (Finkel & Holbrook, 2000). As testosterone is associated with increased metabolic rates (e.g. Buchanan *et al.*, 2001), high testosterone levels (as required for sexual signalling) might also promote oxidative stress. A recent study showed that male zebra finches (*Taeniopygia guttata*) treated with testosterone had a reduced ability to fight off free radicals (Alonso-Alvarez *et al.*, 2007). In this way, the handicap principle was again reformulated as the 'oxidation handicap hypothesis', which suggests that only high-quality males should be able to afford the oxidative

Correspondence: C. Alonso-Alvarez, Instituto de Investigación en Recursos Cinegéticos, IREC (CSIC, UCLM, JCCM), Ronda de Toledo, s/n 13071, Ciudad Real, Spain.
Tel.: +34 926295450; fax: +34 926295451; e-mail: carlos.alonso@uclm.es

challenge promoted by testosterone (Alonso-Alvarez *et al.*, 2007).

All these testosterone properties suggest that this hormone can play a regulatory role in the trade-off between reproductive investment and self-maintenance (Hau, 2007). The currency of such a trade-off could be energy, but also specific nutrients (Peters, 2007). In the first case, part of the energy budget necessary for homeostasis would be diverted to sustain the increase in activity induced by testosterone (e.g. territorial and courtship displays). In the second case, specific molecules required in self-maintenance would also be used as materials for the production of secondary sexual traits controlled by testosterone. Carotenoids could be prime candidates to play such a role.

In addition to their function as pigments in sexual signals in different taxa (i.e. many yellow–red traits), carotenoids play a role in the antioxidant machinery (von Schantz *et al.*, 1999; Hõrak *et al.*, 2007; but see Hartley & Kennedy, 2004). As with testosterone, carotenoids have been associated with the evolutionary trade-off theory. They are considered to be limiting resources allocated to sexual signalling and/or to self-maintenance (von Schantz *et al.*, 1999). The challenge, however, is to understand how both testosterone and carotenoid-mediated trade-offs are interrelated (Peters, 2007). Recently, it has been shown that males from several avian species increased circulating carotenoid levels when experimentally exposed to high testosterone levels (Blas *et al.*, 2006; McGraw, 2006; McGraw *et al.*, 2006; F. Mougeot & J. Martinez-Padilla, unpublished data, but see Mougeot *et al.*, 2007). This raises the question: could the pro-oxidant action of testosterone explain the up-regulation of circulating carotenoids as a compensatory mechanism to combat free radicals?

Here, we propose the hypothesis that there is a testosterone-regulated trade-off in the allocation of carotenoids between avoidance of oxidative stress and colour expression. On the basis of previous avian studies (Blas *et al.*, 2006; McGraw *et al.*, 2006), we predict that high blood testosterone levels increases the availability of the limited resource (carotenoids), maintaining high levels of these pigments in plasma. The question arises about the allocation of carotenoids to both branches of the trade-off. Two different scenarios were predicted where high testosterone levels: (1) favours colour expression at the cost of increased oxidative damage and/or drop of antioxidant levels; or alternatively (2) promote the maintenance of oxidative status (i.e. no change in oxidative damage and maintenance or even increase in antioxidant levels), but at the cost of reducing colour expression. Here, we must mention that circulating levels of antioxidants can increase in the presence of free radicals as an active response to avoid oxidative damages. This pattern has been reported in the case of glutathione (GSH; e.g. Caro & Cederbaum, 2004; Lucchi *et al.*, 2005), a tripeptide thiol often considered as the most important intracellular antioxidant (Wu *et al.*, 2004). We also predict that testosterone promotes a body mass decline due to increased energy demands associated with sexual displaying (Owen-Ashley *et al.*, 2004), or, alternatively, a body mass increase due to the anabolic function of this steroid (Lipar & Ketterson, 2000).

To test the hypothesis, we designed an experiment in one of the species where up-regulation of carotenoids by testosterone has been demonstrated (i.e. the red-legged partridge, *Alectoris rufa*; Blas *et al.*, 2006). Male captive partridges were manipulated as controls (C-males) or treated with either exogenous testosterone (T-males), a blocker of testosterone receptors (flutamide; F-males) or an inhibitor of testosterone conversion to oestrogens (1,4,6-androstatriene-3,17-dione; ATD) plus the cited blocker (FA-males; e.g. Schwabl & Kriner, 1991; Moore *et al.*, 2004; Mougeot *et al.*, 2007). With the last two treatments, we aimed to cancel out any testosterone action on the target tissues. In particular, FA treatment aimed to prevent indirect effects derived from the antioxidant properties of oestrogens (Badeau *et al.*, 2005) that may increase as a result of the aromatization of testosterone when testosterone receptors are blocked by flutamide (e.g. Mougeot *et al.*, 2007). Predictions for F- and FA-males should be the opposite to those stated for T-males (above), whereas any difference between these groups (F- and FA-males) should be only due to the antioxidant effect of oestrogens. With this design, we simultaneously analysed, for the first time in any species, both branches of the proposed trade-off, as well as its potential currency (i.e. carotenoids). With regard to oxidative stress, we assessed the lipid peroxidation in red blood cells (proxy of oxidative damage in cell membranes; Aust, 1985), an estimate of the total antioxidant status in plasma (TAS; Miller *et al.*, 1993) and the levels of glutathione in erythrocytes. With regard to the currency, plasma carotenoid levels were determined. Finally, with regard to the colour expression, the intensity of a carotenoid-dependent trait (the red mask) was analysed. This trait is expressed throughout the breeding season, remaining coloured several months after the onset of mating, loosing its intensity towards the end of the reproductive period (Perez-Rodriguez, 2008). This pattern is common in monogamous birds with bi-parental care (Wachtmeister, 2001), such as partridges, and would allow females to continuously assess the mate's quality, accordingly adjusting their reproductive investment (e.g. Velando *et al.*, 2006a). Colours in integuments such as beaks, legs or masks are dynamic, changing rapidly in response to short-term changes in condition in birds (Faivre *et al.*, 2003; Velando *et al.*, 2006a) including partridges (Perez-Rodriguez & Viñuela, 2008). Therefore, males should maintain a certain colour level throughout the breeding season in order to achieve a better fitness outcome.

Materials and methods

Experimental protocol

The study was carried out at the *Dehesa Galiana* experimental facility (Ciudad Real, Spain). It was conducted on captive red-legged partridges that had never bred before, and that were less than 1-year of age. All individuals were fed commercial pelleted food *ad libitum*. Randomly formed pairs ($n = 117$) were placed in outdoor cages ($1 \times 0.5 \times 0.4$ m^3) at ambient temperature and natural photoperiod. Birds were preventively treated with antiparasitic treatments (i.e. sulphaquinoxaline and levamisole) several weeks before the experiment began, and regular sampling of faeces allowed us to assess discarding helminth or coccidian infections during the study.

All males were sampled for blood and weighed on 10 April 2006. Values from these samples were considered as the initial values of the study. Males were subcutaneously implanted 10 days after blood sampling. Both dates coincided with the period when the highest circulating carotenoid levels were expected, when red carotenoid-based ornaments were displayed at the highest intensity and before egg laying began (Perez-Rodriguez, 2008). All males received two subcutaneous implants (40 mm length, 1.47 mm i.d., 1.96 mm o.d.; Silastic tubing, Dow Corning, Midland, MI, USA) on the back. Males were randomly assigned to one of the four treatments. C-males ($n = 30$) received two empty implants. T-males ($n = 29$) received one of the implants filled with testosterone (Steraolids Inc.; Newport, RI, USA) plus the other empty one. F-males ($n = 29$) received an implant filled with flutamide (Sigma-Aldrich, St. Louis, MO, USA) and another empty one. Finally, FA-males ($n = 29$) were treated with an implant filled with flutamide and an implant filled with ATD (Steraloids Inc.). We expected high testosterone levels in FA-males as a consequence of the treatment blocking the negative feedback loop that keeps plasma hormone concentrations in check (e.g. Moore *et al.*, 2004). Nonetheless, these high testosterone levels would not be functional as T-receptors were being blocked by the flutamide. All implants were sealed at both ends with 1 mm of silicon glue (Nusil Technology, Carpinteria, CA, USA). The length of the implant was established on the basis of a previous work in this species (Blas *et al.*, 2006).

Blood samples and body mass were obtained at 25 (intermediate value) and 70 days (final value) after the implant date. The last sampling event corresponded to the end of the breeding season (Perez-Rodriguez, 2008). Blood samples were always taken within 2 min of the removal of a bird from its cage. Samples were stored at 4 °C until centrifugation and plasma extraction (within 10 h). Both plasma and cell fraction (pellet) were frozen at −80 °C until analysis. One T-male escaped and four males died (one T-male, one FA-male and two C-males) over the course of the study. Moreover, two females (from an FA- and F-couple) died, and information from their mates was removed from the data set if collected after the date of death of the female.

Testosterone assays

Hormone titres were determined at the Centre d'Etudes Biologiques de Chizé (France) by using radioimmunoassay. They were determined in one single assay session by following the procedure described in Chastel *et al.* (2003). Testosterone antiserum was kindly provided by Dr Gérald Picaper (Medecine Nucleaire, CHU la Source, Orleans, France). The lowest detectable concentration was 0.1 ng mL^{-1}. Cross-reactivities were less than 15% and 2% between testosterone and dihydrotestosterone, and between testosterone and androstenodione respectively. Recovery rates were higher than 90%. The intra-assay coefficient of variation was 5%.

Oxidative damage

Oxidative damage was assessed by determining the level of lipid peroxidation in erythrocytes, following Aust (1985). The principle of the test is based on the fact that most tissues contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes, the concentrations of which increase due to oxidative stress. Briefly, the blood pellet was thawed and the red blood cells were pipetted whilst avoiding the pellet surface (i.e. the buffy coat containing white blood cells). Erythrocytes were immediately diluted (1 : 10 w/v) and homogenized in a stock buffer (0.01 M PBS and 0.02 M EDTA) while working on ice to avoid oxidation. One millilitre of the homogenate was mixed with 2 mL of a solution (15% trichloroacetic acid, 0.25 N HCl and 0.375% thiobarbituric acid in H_2O) and with 20 μL of diluted BHT [2% BHT (2,6-di-tert-butyl-4-methylphenol) in ethanol] in a closed glass tube. Tubes were then warmed for 30 min at 90 °C and then cooled with ice-cold water (10 min). The absorbance of the supernatant was then determined by using spectrophotometry at 535 nm after centrifuging at 2025 *g* for 15 min. Concentrations of peroxidized lipids were determined by comparing readings against those obtained from a calibration curve created with 0, 1.25, 2.50 and 5 nmol/mL of malondialdehyde (MDA; i.e. the end product of lipid peroxidation) in H_2O. Concentrations of peroxidized lipids per gram of pellet are expressed in nmol MDA per gram. Repeatability (Lessells & Boag, 1987) was estimated on a subset of samples ($r = 0.80$, $P < 0.04$, $n = 20$).

Total glutathione

Total glutathione levels (also known as tGSH) in erythrocytes were determined using the method described by Griffith (1980) with some particular

modifications. Briefly, three working solutions were made up in the same stock buffer as follows: (I) 0.3 mM NADPH; (II) 6 mM DTNB; and (III) 50 units of glutathione reductase per mL. An aliquot (0.4 mL) of the same homogenate used for the TBARS test was vortexed (three times, 5 s each time, within a 15-min period) with 0.5 mL of diluted tricholoroacetic acid (10% in H_2O) and 0.1 mL of stock buffer. The addition of stock buffer allowed a best fit to the standard curve. In between each vortex, the samples were stored refrigerated in the dark to prevent oxidation. Subsequently, the mixture was centrifuged at 1125 *g*, for 15 min at 6 °C, and the supernatant removed. Then, the following steps were carried out in an automated spectrophotometer (A25-Autoanalyzer; Biosystems SA, Barcelona, Spain). Solutions I and II were mixed at 7 : 1 by volume; then 160 μL of this new mixture was automatically added to 40 μL of sample (the supernatant noted above) in a cuvette. After 15 s, 20 μL of solution III was added. The absorbance at 405 nm was monitored after 30 and 60 s. The change in absorbance was used to determine the total glutathione concentration in red blood cells by comparing the output with the results from a standard curve generated by serial dilution of glutathione from 1 to 0.031 mM. Repeatability was determined using a subset of 20 samples, measured twice ($r = 0.81$, $P < 0.001$). Results are given in mmol g^{-1} of pellet.

Antioxidant status

The total antioxidant status in plasma (TAS; Miller *et al.*, 1993) was assessed by means of commercial kits (Randox Laboratories Ltd, Crumlin, UK) adapted for use in an automated spectrophotometer (A25-Autoanalyzer; Biosystems SA). Plasma samples were incubated for 15 s with a chromogen composed of metmyoglobin and 2,2-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS). Then, hydrogen peroxide was added and the sample was incubated for 195 s. Addition of hydrogen peroxide induces the production of the radical cation ABTS, which generates a blue-green colour. The intensity of this colour was measured at 600 nm before and after the addition of H_2O_2, i.e. to determine the change in the intensity of the colour. Antioxidants in plasma cause suppression of this colour change to a degree which is proportional to their concentration. Repeatability was confirmed using a random subset of samples measured twice ($r = 0.94$, $P < 0.001$, $n = 20$). Results are given as mmol L^{-1} of total antioxidants.

Circulating carotenoids

Total carotenoid concentration in plasma was determined by using spectrophotometry, utilizing a standard curve generated with lutein (Sigma), and after verification by using HPLC that lutein was the predominant carotenoid in the samples (J. Blas and J. Garrido, personal communication). Plasma aliquots (60 μL) were diluted in acetone (1 : 10) and mixed. The flocculent protein was precipitated by centrifuging at 11 000 *g* for 10 min. The absorbance of the supernatant was determined at 446 nm. Carotenoid values measured twice on a subsample were repeatable ($r = 0.99$, $P < 0.001$, $n = 20$).

Carotenoid nature of the red mask

The carotenoid composition of the red mask was confirmed by an ongoing research by using HPLC on samples of this tissue from several killed partridges (R. Mateo, unpublished data).

Colour assessment

Digital pictures of red masks were taken at each sampling event under standardized light conditions, with a standard grey chip (Kodak, New York, NY, USA) placed close to the bird. Colour intensity was measured on pictures using Adobe Photoshop 7.0. Analyses were performed by the same person who was blind to the identity of the bird. The redness of the red mask (bare lore and eye ring; Blas *et al.*, 2006) was determined by selecting its surface area on the picture, then by recording the mean values of red, green and blue colour components (RGB system). Mean RGB values obtained per duplicate were repeatable ($r = 0.82$, $P < 0.001$), and therefore, average values were used. Hue values were obtained from mean RGB using the algorithm described in Foley & Van Dam (1984). The hue of the reference chip was used as a covariate in models in order to control for possible subtle changes in lighting conditions, although its effect was never significant (all $P > 0.10$). High values of hue indicated less redness.

Statistical analyses

Repeated-measurement mixed models (PROC MIXED, SAS software; SAS Institute, 2001) were used to test the effect of the treatment (a four-level fixed factor) on the dependent variables throughout the study (sampling time × treatment interaction). Body mass and carotenoids were also tested as covariates in the models, being removed when they were nonsignificant ($P > 0.05$). Bird identity was included as a random factor. As some birds died or escaped before the end of the study, and because some blood samples could not be analysed due to haemolysis or lack of volume, a Satterthwaite correction was used to approximate the degrees of freedom. Tukey's *post hoc* tests were used for pairwise comparisons, *P*-values being provided in the Results section. Testosterone was log-transformed to meet the normality assumption. Initial values of any parameter (testosterone, total carotenoids, lipid peroxidation, total glutathione, TAS, hue and body mass) did not differ between the groups that further received any of the hormone treatments (all $P > 0.140$).

Results

Hormonal phenotypes

Testosterone levels at the initial sampling (nonmanipulated birds) ranged from 0.1 to 5.82 ng mL^{-1}, which is within the range previously reported in this species (Bottoni *et al.*, 1993). Afterwards, T-males showed the highest mean values at both the intermediate (2.68, 0.76, 0.57 and 4.45 ng mL^{-1} for FA-, F-, C- and T-males respectively) and final sampling events (1.32, 0.67, 0.48 and 3.98 ng mL^{-1}). The time × treatment interaction was highly significant ($F_{6,220} = 23.52$, $P < 0.0001$). FA-males showed testosterone levels that were higher than those found in both C- and F-males (all *post hoc* $P < 0.001$), but still significantly lower than those found in T-males (all $P < 0.012$). Testosterone concentrations in C- and F-males never differed (all $P > 0.14$).

Body mass changes

Mean body mass of T-males significantly increased during the experiment (449.3, 451.4 and 458.1 g, for initial, intermediate and final samplings respectively), whereas birds in other groups lost weight at the end of the study (mean loss: 13.14, 7.06 and 13.57 g for FA-, F- and C-males respectively). This was reflected in a significant treatment × time interaction (Table 1).

The potential currency of the trade-off: circulating carotenoids

Circulating carotenoid levels were correlated with the hue at the beginning of the experiment (pre-implanting values: $r = -0.633$, $P < 0.001$), indicating that the redder the colour, the larger the amount of carotenoids in plasma.

Afterwards, carotenoid levels regularly decreased throughout the study (Table 1), but T-males showed a slower decline (Fig. 1). Hence, final values for T-males were higher than that for the other groups (all *post hoc* $P < 0.035$). Final values of non-T-treated males did not differ among them (all $P > 0.19$). The time × treatment interaction remained significant ($P < 0.05$) when body mass was added as a covariate ($F_{1,139} = 5.19$, $P = 0.024$; estimated slope ± SE: +0.014 ± 0.006), which could discard a potential bias due to differential food intake.

To explore whether circulating carotenoids were related to the antioxidant machinery, carotenoid levels were also tested as a covariate in the models testing lipid peroxidation, glutathione and TAS variability (below). Carotenoids did not influence the variability in the first two parameters (both $P > 0.20$). However, a significant positive correlation between TAS and carotenoids ($F_{1,314} = 8.59$, $P = 0.004$; estimated slope ± SE: +0.012 ± 0.004) was detected.

Testosterone effects on oxidative status

Levels of lipid peroxidation in erythrocytes did not differ between treatments during the study, suggesting an absence of differences in oxidative damage (Table 1; Fig. 1). The time factor showed a significant effect (Table 1) because there was a significant increase between the initial and intermediate values (*post hoc* $P = 0.01$).

With regard to the antioxidant machinery, the levels of total glutathione were significantly affected by both time and the time × treatment interaction (Table 1; Fig. 1). C-males presented stable values (all comparisons between sampling events: $P > 0.20$), whereas T-males increased glutathione levels between the first two sampling events (*post hoc* $P = 0.017$). Levels decreased again between the intermediate and final measurements ($P = 0.014$). F- and FA-males did not show significant changes between the initial and intermediate sampling points ($P > 0.28$), but levels did decrease between the intermediate and final measurements (both $P < 0.027$). Meanwhile, TAS was affected by sampling time (levels declined during the study), but was not significantly influenced by the treatment (Table 1; Fig. 1).

Testosterone effects on colour expression

The hue of the red mask increased throughout the experiment (time factor: $F_{2,257} = 67.16$, $P < 0.0001$),

Table 1 Repeated-measurement mixed models testing the effect of the hormonal manipulation on several parameters throughout the experiment.

	Treatment			Time			Treatment × time		
Dependent variables	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Body mass	1.39	3, 117	0.248	12.42	2, 219	<0.0001	7.77	6, 219	<0.0001
Total plasma carotenoids	3.86	3, 103	0.012	55.9	2, 193	<0.0001	2.48	6, 193	0.025
TAS	0.90	3, 114	0.443	4.36	2, 208	0.014	1.75	6, 208	0.112
Total glutathione	0.51	3, 99.7	0.678	11.68	2, 177	<0.0001	2.32	6, 180	0.035
Lipid peroxidation	0.40	3, 103	0.754	5.05	2, 166	0.007	0.84	6, 168	0.539

The identity of each individual was included as a random factor in every model (all $P < 0.02$).

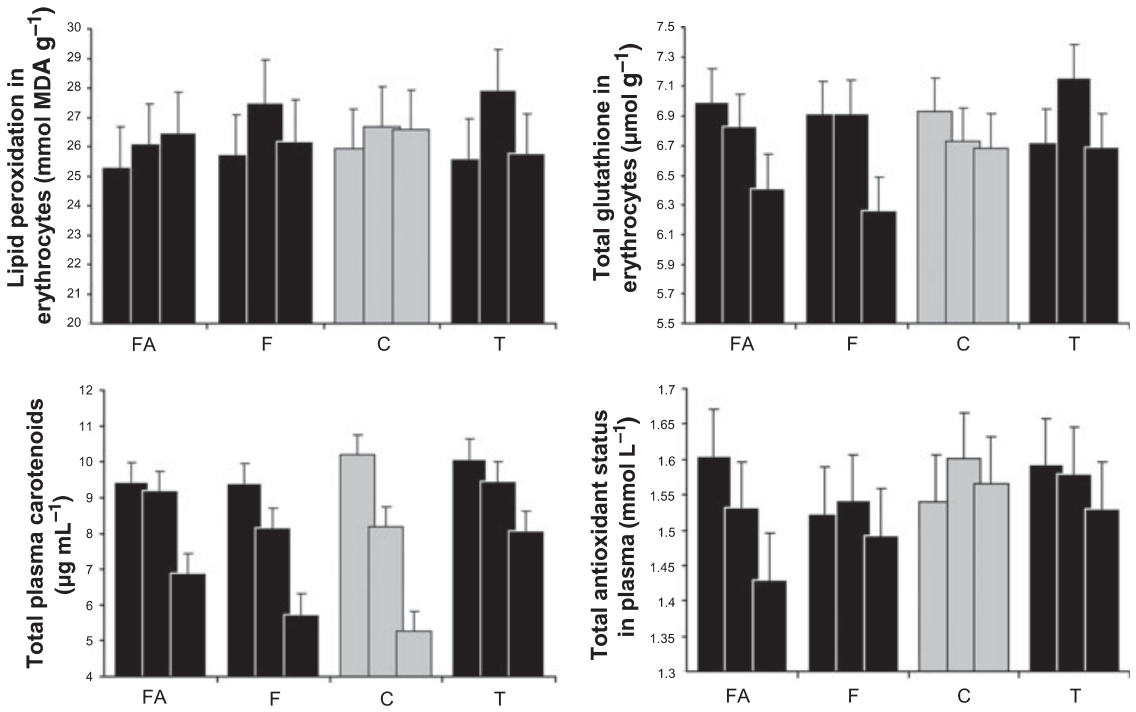

Fig. 1 Lipid peroxidation and level of total glutathione in erythrocytes, total antioxidant status in plasma (TAS) and level of plasma carotenoids in male partridges treated with testosterone antagonists (FA and F; see Introduction), manipulated as controls (C) or treated with exogenous testosterone (T). From left to right, each set of bars shows the initial, intermediate and final values throughout the study. Graph shows the least-squares mean ± SE, from mixed models which included the bird identity as a random factor.

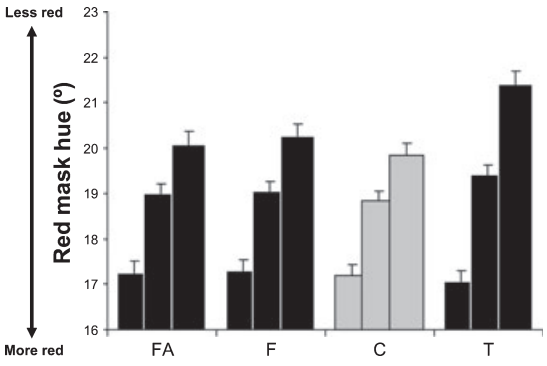

Fig. 2 Changes in the hue of the red mask of male partridges treated with androgen antagonists (FA and F; see Introduction), manipulated as controls (C) or treated with testosterone (T). From left to right, each set of bars shows the initial, intermediate and final values throughout the study. Graph shows the least-squares mean ± SE, from mixed models which included the bird identity as a random factor.

indicating a regular loss of redness (Fig. 2). However, T-males showed a steeper increase, compared with the other three groups (treatment: $F_{3,112} = 4.92$, $P = 0.003$; treatment × time: $F_{6,222} = 4.27$, $P = 0.0004$). T-birds showed less red masks than control birds at the intermediate sampling point (*post hoc* $P = 0.046$), and had the palest trait among the four groups at the end of the experiment (all $P < 0.006$). There were no differences in hue among non-T-treated groups during the study (all $P > 0.15$).

Integrating the trade-off

To look deeper into the role played by testosterone in the trade-off between colour expression and avoidance of oxidative stress, we carried out ANCOVA models testing the interaction between treatment and each index of oxidative status on the final measure of redness. Lipid peroxidation showed a significant interaction with the treatment ($F_{3,100} = 3.62$, $P = 0.016$; initial hue as a covariate: $F_{1,100} = 43.84$, $P < 0.001$; Fig. 3). In this way, T-males showed a negative relationship between hue and oxidative damage ($r = -0.42$, $P = 0.030$), whereas C-males showed the opposite ($r = 0.39$, $P = 0.048$). FA- and F-males did not show any significant pattern (both $P > 0.12$). Glutathione and TAS ANCOVAS did not present such significant interaction (both $P > 0.20$).

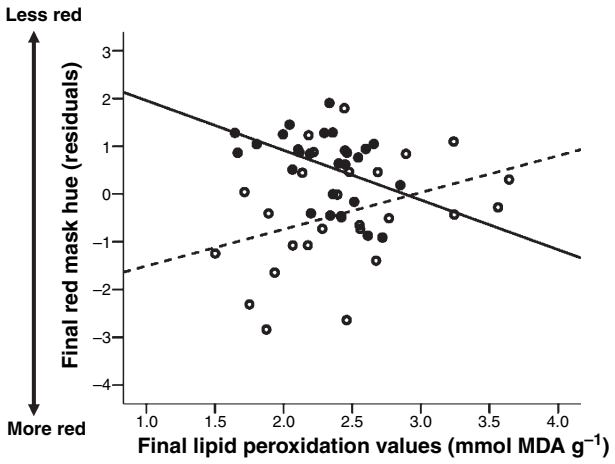

Fig. 3 The relationship between mask redness and oxidative damage (lipid peroxidation) at the end of the experiment differed between testosterone-treated males (full dots, solid line) and controls (empty dots, dotted line). Values in *y*-axis are residuals from a regression between the final and initial hue values. The relationship was not significant in birds treated with antiandrogens and hence they are not showed.

Discussion

These results support one of the scenarios predicted in the Introduction, i.e. that high testosterone levels promote the maintenance of the oxidative status, at the cost of reducing colour expression. Birds with increased T-levels maintained a higher level of circulating carotenoids, but they showed a reduction in mask redness. Furthermore, T-males did not show the expected negative impact of sustaining high T-levels (see Alonso-Alvarez *et al.*, 2007), as they did not present increased lipid peroxidation. However, those T-males maintaining higher colour expression paid the cost in terms of increased oxidative damage (Fig. 3). Hence, birds treated with high T-levels seem to have solved the proposed trade-off by sacrificing colour expression, allowing increased carotenoid availability in blood and the avoidance of the oxidative damage.

Nonetheless, the allocation pattern should plastically change when availability and/or resource demands change (Zera & Harshman, 2001). Results from recent studies, in which at least one branch of the trade-off (i.e. colour expression) and the resource (i.e. circulating carotenoids) were experimentally assessed, support this view (Blas *et al.*, 2006; McGraw, 2006; McGraw *et al.*, 2006; Mougeot *et al.*, 2007). The finding that males which sustained high T-levels also had higher blood carotenoid levels is mostly consistent among studies. However, F. Mougeot & J. Martinez-Padilla (unpublished data) recently found increased carotenoid levels in response to T-treatment in red grouse (*Lagopus lagopus*) males from some populations, but not in others, perhaps because of an interaction with social factors (i.e. population density). Meanwhile, the investment in colour expression of T-males differed between studies, either showing an increase (McGraw *et al.*, 2006), a decrease (as in our study) or no change at all (Blas *et al.*, 2006). Finally, in fishes, Kurtz *et al.* (2007) showed that male three-spined sticklebacks (*Gasterosteus aculeatus*) treated with androgens, increased colour expression at the cost of a trend towards higher oxidative damage. However, in that study, changes in circulating carotenoids were not assessed.

In our experiment, accelerated colour decline in T-males may be explained at both the proximate and ultimate levels. In the first case, it could be due to increased demand for carotenoids to buffer free radicals. The contrasted relationship between colour and lipid peroxidation in T- and C-birds at the end of the study supports this view (Fig. 3). Interestingly, Siitari *et al.* (2007) recently reported a T-mediated decrease in the redness of a very similar trait (the red supraorbital comb) in another gallinacean (the black grouse; *Tetrao tetrix*). These authors also suggested that the higher production of free radicals promoted by the experimental increase in androgen levels could explain this finding. Meanwhile, at the ultimate level, decreased colour expression could be interpreted in terms of a life-history trade-off. Individuals from iteroparous species must trade current vs. future reproductive investments in order to maximize their lifetime fitness (Williams, 1966). Captive red-legged partridges can successfully breed until at least 8 years old (C. Alonso-Alvarez, unpublished data). Yearlings (as used in this study) faced with an oxidative challenge (as for T-males) should avoid sacrificing resources in carotenoid-dependent traits if this reduces survival and future breeding opportunities. This strategy would be particularly suitable when carotenoid availability in blood decreases (i.e. throughout the post-mating period). The resource allocation rule may, however, change in subsequent years, promoting the investment in sexual signalling when future breeding prospects decline (Velando *et al.*, 2006b).

In spite of the pale colour acquired, T-males showed the slowest decrease in plasma carotenoid levels. As proposed, higher plasma carotenoid levels in these birds could be the result of an active response against the oxidative challenge promoted by testosterone (Alonso-Alvarez *et al.*, 2007). The pattern of change showed by total glutathione supports this last view. Glutathione is one of the most important intracellular antioxidants, and is present in virtually all cells of vertebrates (Wu *et al.*, 2004). An increase in glutathione blood levels in response to an oxidative challenge has been broadly reported in the literature (Radice *et al.*, 1998; Jimenez-Cervantes *et al.*, 2001; Caro & Cederbaum, 2004; Lucchi *et al.*, 2005). Moreover, it has been reported that castration decreases glutathione production in rats, whereas testosterone replacement restores their levels (Singhal & Vijayvargiya, 1983). We have also found that senescent red-legged partridges present high levels of both total

glutathione and lipid peroxidation in erythrocytes (C. Alonso-Alvarez, unpublished data), perhaps as a result of aging-related oxidative stress. In summary, the changes in glutathione concentration detected in the present study seem to support the pro-oxidant action of testosterone and the oxidation handicap hypothesis (Alonso-Alvarez *et al.*, 2007).

However, the role of carotenoids as antioxidant resources in the proposed trade-off can be questioned. In a meta-analysis on recent literature, Costantini & Møller (2008) showed that the relation between circulating carotenoids and antioxidant power or oxidative damage among six avian species was weak. Here, circulating carotenoids were positively related to TAS throughout the experiment and a similar result was found in an independent sample of 288 red-legged partridges (C. Alonso-Alvarez, unpublished data). In both cases, the relationship was weak, but nevertheless, statistically significant. Costantini & Møller (2008) speculated that the predominant role of carotenoids may be immunostimulation (see also Hartley & Kennedy, 2004). As testosterone can play an immunosuppressive role (Folstad & Karter, 1992; Roberts *et al.*, 2004), we cannot dismiss the fact that higher carotenoid levels observed in T-treated partridges and other bird species may be the consequence of a resource mobilization to combat pathogens. Nevertheless, current knowledge suggests that, although some mechanisms may link carotenoids to immunity independently of oxidation (i.e. regulation of membrane fluidity, gap-junctional communication and certain intracellular signalling pathways; Chew & Park, 2004; Palozza *et al.*, 2006), many other mechanisms directly or indirectly (through intracellular redox) relate the immune-enhancing function of carotenoids to their role as free radical quenchers (Chew & Park, 2004; Palozza *et al.*, 2006). Moreover, we noted that T-treated males were the only group to gain body mass during the study, which is not coherent with immunossuppression or any associated disease. Instead, such a body mass gain could be related to the anabolic action of androgens (Lipar & Ketterson, 2000), which supports the hypothesis of an increased demand for antioxidants.

To conclude, our results suggest that testosterone is able to promote an oxidative challenge that can be effectively controlled. Control depends on the sacrifice of one of the arms of the proposed trade-off, i.e. sexual signalling. The fact that T-males maintaining redder masks paid the effort in terms of increased oxidative damage, whereas C-males showed the opposite pattern (Fig. 3), supports the presence of the trade-off. Moreover, the maintenance of higher carotenoid levels in individuals sustaining high testosterone levels suggests a T-mediated allocation of these pigments to fight off oxidative stress. However, we must be cautious in this interpretation as no direct link between carotenoids and oxidative damage was established (not significant correlation between carotenoid levels and lipid peroxidation). Furthermore, the use of carotenoids in actions unrelated to the combat of free radicals (i.e. certain immune pathways) cannot be dismissed. It is now necessary to broaden the perspective, and integrate immune function as an additional arm into the same trade-off.

Acknowledgments

We are grateful to Carlos Cano and Francisco Perez from Consejeria de Medio Ambiente, Junta de Comunidades de Castilla-La Mancha (JCCM), Spain, for kindly supplying partridges for the study. Thanks to Emiliano Sobrino Fernando Dueñas and Luis Montó for bird maintenance and Amalia Molinero and Clara Rico for technical support at the lab and field facilities, respectively. Thanks to Mark A. Taggart, for reviewing the English language. Thanks to Patrick Fitze for advice on colour measurement and to C. Trouvé and A. Lacroix for their assistance on the hormone assays. We are grateful to Gary R. Bortolotti, François Mougeot, Gabriele Sorci and Alberto Velando for reviewing previous versions of the manuscript. C. A.-A. and L. P.-R. were funded by a Ramon y Cajal Fellowship (Ministerio de Educación y Ciencia, Spain) and by a postdoctoral contract from the JCCM, respectively. The study was funded by the JCCM (project PAI-06-0018) and the Ministerio de Educación y Ciencia (project CGL2006-10357-C02-02), Spain.

References

Alonso-Alvarez, C., Bertrand, S., Faivre, B., Chastel, O. & Sorci, G. 2007. Testosterone and oxidative stress: the oxidation handicap hypothesis. *Proc. R. Soc. Lond. B* **274**: 819–825.

Aust, S.D. 1985. Lipid peroxidation. In: *Handbook of Methods for Oxygen Radical Research* (R.A. Greenwald, ed.), pp. 203–207. CRC Press, Boca Raton, FL.

Badeau, M., Adlercreutz, H., Kaihovaara, P. & Tikkanen, M.J. 2005. Estrogen A-ring structure and antioxidative effect on lipoproteins. *J. Steroid Biochem.* **96**: 271–278.

Blas, J., Perez-Rodriguez, L., Bortolotti, G.R., Viñuela, J. & Marchant, T.A. 2006. Testosterone increases bioavailability of carotenoids: insights into the honesty of sexual signalling. *Proc. Natl Acad. Sci. USA.* **103**: 18633–18637.

Bottoni, L., Massa, R., Lea, R.W. & Sharp, P.J. 1993. Mate choice and reproductive success in the red-legged partridge (*Alectoris rufa*). *Horm. Behav.* **27**: 308–317.

Buchanan, K.L., Evans, M., Goldsmith, A.R., Bryant, D.M. & Rowe, L.V. 2001. Testosterone influences basal metabolic rate in male house sparrows: a new cost of dominance signalling? *Proc. R. Soc. Lond. B* **268**: 1337–1344.

Caro, A.A. & Cederbaum, A.I. 2004. Oxidative stress, toxicology, and pharmacology of CYP2E1. *Annu. Rev. Pharmacol.* **44**: 27–42.

Chastel, O., Lacroix, A. & Kersten, M. 2003. Pre-breeding energy requirements: thyroid hormone, metabolism and the timing of reproduction in house sparrows *Passer domesticus*. *J. Avian Biol.* **34**: 298–306.

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

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

Faivre, B., Gregoire, A., Preault, M., Cezilly, F. & Sorci, G. 2003. Immune activation rapidly mirrored in a secondary sexual trait. *Science* **300**: 29–31.

Finkel, T. & Holbrook, N.J. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* **408**: 239–247.

Foley, J.D. & Van Dam, A. 1984. *Fundamentals of Interactive Computer Graphics*. Addison-Wesley, Reading, MA.

Folstad, I. & Karter, A.K. 1992. Parasites, bright males and the immunocompetence handicap. *Am. Nat.* **139**: 603–622.

Griffith, O.W. 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinilpiridine. *Anal. Biochem.* **106**: 207–212.

Hartley, R.C. & Kennedy, M.W. 2004. Are carotenoids a red herring in sexual display? *Trends Ecol. Evol.* **19**: 353–354.

Hau, M. 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. *BioEssays* **29**: 133–144.

Hõrak, P., Saks, L., Zilmer, M., Karu, U. & Zilmer, K. 2007. Do dietary carotenoids alleviate the cost of immune activation? An experiment with greenfinches *Am. Nat.* **170**: 625–635.

Jimenez-Cervantes, C., Martínez-Esparza, M., Pérez, C., Daum, N., Solano, F. & García-Borrón, J.C. 2001. Inhibition of melanogenesis in response to oxidative stress: transient downregulation of melanocyte differentiation markers and possible involvement of microphthalmia transcription factor. *J. Cell Sci.* **114**: 2335–2344.

Kurtz, J., Kalbe, M., Langefors, S., Mayer, I., Milinski, M. & Hasselquist, D. 2007. An experimental test of the immunocompetence handicap hypothesis in a teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks. *Am. Nat.* **170**: 509–519.

Lessells, C.M. & Boag, P.T. 1987. Unrepeatable repeatabilities: a common mistake. *Auk* **104**: 116–121.

Lipar, J.L. & Ketterson, E.D. 2000. Maternally derived yolk testosterone enhances the development of the hatching muscle in the red-winged blackbird *Agelaius phoeniceus*. *Proc. R. Soc. Lond. B* **267**: 2005–2010.

Lucchi, L., Bergamini, S., Iannone, A., Perrone, S., Stipo, L., Olmeda, F., Caruso, F., Tomasi, A. & Albertazzi, A. 2005. Erythrocyte susceptibility to oxidative stress in chronic renal failure patients under different substitutive treatments. *Artif. Organs* **29**: 67–72.

Maynard Smith, J. & Harper, D. 2003. *Animal Signals*. Oxford University Press, New York.

McGraw, K.J. 2006. Sex steroid dependence of carotenoid-based colouration in female zebra finches. *Physiol. Behav.* **88**: 347–352.

McGraw, K.J., Correa, S.M. & Adkins-Regan, E. 2006. Testosterone upregulates lipoprotein status to control sexual attractiveness in a colourful songbird. *Behav. Ecol. Sociobiol.* **60**: 117–122.

Miller, N.J., Rice-Evans, C.A., Davies, M.J., Gopinathan, V. & Milner, A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **84**: 407–412.

Moore, I.T., Walker, B.G. & Wingfield, J.C. 2004. The effects of combined aromatase inhibitor and anti-androgen on male territorial aggression in a tropical population of rufous-collared sparrows, *Zonotrichia capensis*. *Gen. Comp. Endocrinol.* **135**: 223–229.

Mougeot, F., Perez-Rodriguez, L., Martinez-Padilla, J., Leckie, F. & Redpath, S.M. 2007. Parasites, testosterone and honest carotenoid-based signalling of health. *Funct. Ecol.* **21**: 886–898.

Owen-Ashley, N.T., Hasselquist, D. & Wingfield, J.C. 2004. Androgens and the immunocompetence handicap hypothesis: unraveling direct and indirect pathways of immunosuppression in song sparrows. *Am. Nat.* **164**: 490–505.

Palozza, P., Serrín, S. & Calviello, G. 2006. Carotenoids as modulators of intracellular signaling pathways. *Curr. Signal Transduct. Ther.* **1**: 325–335.

Perez-Rodriguez, L. 2008. Carotenoid-based ornamentation as a dynamic but consistent individual trait. *Behav. Ecol. Sociobiol.* **62**: 995–1005.

Perez-Rodriguez, L. & Viñuela, J. 2008. Carotenoid-based bill and eye ring coloration as honest signals of condition: an experimental test in the red-legged partridge (*Alectoris rufa*). *Naturwissenschaften*. doi: 10.1007/s00114-008-0389-5.

Peters, A. 2007. Testosterone and carotenoids: an integrated view of trade-offs between immunity and sexual signalling. *BioEssays* **29**: 427–430.

Radice, S., Marabini, L., Gervasoni, M., Ferraris, M. & Chiesara, E. 1998. Adaptation to oxidative stress: effects of vinclozolin and iprodione on the HepG2 cell line. *Toxicology* **129**: 183–191.

Roberts, M.L., Buchanan, K.L. & Evans, M.R. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. *Anim. Behav.* **68**: 227–239.

SAS Institute. 2001. SAS/STAT Software: Changes and Enhancements, Version 8.2. SAS Publishing, Cary, NC.

von Schantz, T.V., Bensch, S., Grahn, M., Hasselquist, D. & Wittzell, H. 1999. Good genes oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. Lond. B* **266**: 1–12.

Schwabl, H. & Kriner, E. 1991. Territorial aggression and song of male European robins (*Erithacus rubecula*) in autumn and spring: effects of antiandrogen treatment. *Horm. Behav.* **25**: 180–194.

Siitari, H., Alatalo, R.V., Halme, P., Buchanan, K.L. & Kilpimaa, J. 2007 Color signals in the black grouse (*Tetrao tetrix*): signal properties and their condition dependency. *Am. Nat.* **169**, S81–S92, Suppl.

Singhal, R.L. & Vijayvargiya, R. 1983. Studies on glutathione metabolism in ventral prostate and chemically induced prostatic carcinoma in rats. *Biosci. Rep.* **3**: 241–253.

Velando, A., Beamonte, R. & Torres, R. 2006a. Pigment-based skin colour in the blue-footed booby: an honest signal of current condition used by females to adjust reproductive investment. *Oecologia* **149**: 543–552.

Velando, A., Drummond, H. & Torres, R. 2006b. Senescent birds redouble reproductive effort when ill: confirmation of the terminal investment hypothesis. *Proc. R. Soc. Lond. B* **273**: 1443–1448.

Wachtmeister, C.A. 2001. Display in monogamous pairs: a review of empirical data and evolutionary explanations. *Anim. Behav.* **61**: 861–868.

Williams, G.C. 1966. Natural selection, the costs of reproduction, and a refinement of Lack's principle. *Am. Nat.* **100**: 687–690.

Wu, G., Fang, Y.Z., Yang, S., Lupton, J.R. & 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**: 205–213.

Zera, A.J. & Harshman, L.G. 2001. The physiology of life history trade-offs in animals. *Annu. Rev. Ecol. Syst.* **32**: 95–126.

Received 6 February 2008; revised 13 June 2008; accepted 13 June 2008