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Oxidative stress and the effect of parasites on a carotenoid-based ornament

F. Mougeot[1,2,*], J. Martínez-Padilla[3], J. D. Blount[4], L. Pérez-Rodríguez[2,3], L. M. I. Webster[3] and S. B. Piertney[3]

[1]Estación Experimental de Zonas Áridas, CSIC, General Segura 1, 04001 Almeria, Spain, [2]Instituto de Investigación en Recursos Cinegéticos, IREC (CSIC, UCLM, JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain, [3]School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK and [4]Centre for Ecology and Conservation, School of Biosciences, University of Exeter, Cornwall Campus, Penryn TR10 9EZ, UK

*Author for correspondence (francois.mougeot@eeza.csic.es)

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SUMMARY

Oxidative stress, the physiological condition whereby the production of reactive oxygen and nitrogen species overwhelms the capacity of antioxidant defences, causes damage to key bio-molecules. It has been implicated in many diseases, and is proposed as a reliable currency in the trade-off between individual health and ornamentation. Whether oxidative stress mediates the expression of carotenoid-based signals, which are among the commonest signals of many birds, fish and reptiles, remains controversial. In the present study, we explored interactions between parasites, oxidative stress and the carotenoid-based ornamentation of red grouse *Lagopus lagopus scoticus*. We tested whether removing nematode parasites influenced both oxidative balance (levels of oxidative damage and circulating antioxidant defences) and carotenoid-based ornamentation. At the treatment group level, parasite purging enhanced the size and colouration of ornaments but did not significantly affect circulating carotenoids, antioxidant defences or oxidative damage. However, relative changes in these traits among individuals indicated that males with a greater number of parasites prior to treatment (parasite purging) showed a greater increase in the levels of circulating carotenoids and antioxidants, and a greater decrease in oxidative damage, than those with initially fewer parasites. At the individual level, a greater increase in carotenoid pigmentation was associated with a greater reduction in oxidative damage. Therefore, an individual's ability to express a carotenoid-based ornament appeared to be linked to its current oxidative balance and susceptibility to oxidative stress. Our experimental results suggest that oxidative stress can mediate the impact of parasites on carotenoid-based signals, and we discuss possible mechanisms linking carotenoid-based ornaments to oxidative stress.

Key words: lipid peroxidation, oxidative damage, antioxidant defences, honest signalling, nematode *Trichostrongylus tenuis*, red grouse *Lagopus lagopus scoticus*, sexual selection.

INTRODUCTION

Many of the red–yellow ornaments used by animals as social signals or for facilitating mate choice are pigmented by carotenoids (Hill, 2002; Hill and McGraw, 2006). There is considerable interest in understanding the proximate and ultimate causes of variability in such carotenoid-based displays (Hill and McGraw, 2006; Olson and Owens, 1998; Olson and Owens, 2005), with particular emphasis on sexually selected traits and on the physiological mechanisms constraining or enhancing carotenoid allocation to ornaments to ensure honest signalling (Hill and McGraw, 2006). In birds, the production costs of plumage colouration have received particular attention (e.g. Badyaev and Hill, 2000; Hill and Brawner, 1998; Shawkey and Hill, 2005), as well as the carotenoid-based colouration of integument or beak displays, which, unlike plumage, can change rapidly and could provide a useful indicator of current health and parasite infections (e.g. Blount et al., 2003; Bortolotti et al., 2009; Faivre et al., 2003; Mougeot et al., 2007b).

Carotenoid pigments have been suggested to mediate the honesty of signals through allocation trade-offs with other traits such as the immune system and with reproduction (Blount, 2004). Carotenoids have immuno-stimulant properties (Blount et al., 2003; Møller et al., 2000; McGraw and Ardia, 2003), so individuals may trade allocation of available carotenoids to their ornaments or towards self maintenance and immune function (von Schantz et al., 1999). The carotenoid-based ornaments of birds are particularly sensitive to parasite infections (Lozano, 1994), as demonstrated with a range of diseases and parasites (e.g. Brawner et al., 2000; Hill et al., 2004; Hõrak et al., 2001; Hõrak et al., 2004; Martinez-Padilla et al., 2007), and should also be sensitive to oxidative stress (Alonso Alvarez et al., 2008; Pike et al., 2007a; von Schantz et al., 1999), itself intimately linked with immune function and disease or parasite resistance (Dowling and Simmons, 2009; Hõrak et al., 2007; Romero et al., 1998).

Producing an immune response involves the release of reactive oxygen and nitrogen species (ROS and RNS, respectively) that could be harmful to the host, and which may impose a significant cost of immunity, although selection is expected to act to minimise such costs (von Schantz et al., 1999; Dowling et al., 2009). Immune system activation in response to viral or parasite infections produces ROS/RNS to help counter invading pathogens; however, their overproduction can lead to oxidative stress when the production of ROS/RNS overwhelms the capacity of antioxidant defences (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 2007). ROS/RNS are produced by oxidation and reduction reactions, and are unstable, very reactive by-products of normal metabolic processes that can cause extensive damage to cellular proteins, lipids and DNA, particularly when individuals lack sufficient antioxidant protection (Halliwell et al., 2007; Splettstoesser and Schuff-Werner, 2002; von Schantz et al., 1999). Carotenoids can scavenge free radicals and cytotoxic molecules produced during immune response (Chew and Park, 2004) and can thereby reduce auto-reactivity and self-harm (von Schantz et al., 1999). Immune system activation consumes

carotenoids (e.g. Perez-Rodriguez et al., 2008) whereas carotenoid supplementation can increase immune responsiveness (McGraw and Ardia, 2003; Blount et al., 2003) and thereby alleviate the costs of mounting an immune response (Hõrak et al., 2007). In birds, carotenoids appear as relatively weak antioxidants compared with many other antioxidant compounds (Cohen and McGraw, 2009; Costantini and Møller, 2008; Perez-Rodriguez et al., 2008), so a possible trade-off in carotenoid use for colouration *vs* the maintenance of the oxidative balance should be addressed experimentally (Perez-Rodriguez, 2009).

In the present study, we explored the relationships between parasites, oxidative stress and carotenoid-based signals by asking whether the experimental removal of parasites from a host (and thereby reducing the requirement for raising anti-parasite immune responses) influenced both oxidative balance (levels of oxidative damage and circulating antioxidant defences) and the expression of a carotenoid-based signal. We also investigated whether parasite effects on oxidative balance could mediate the effects of parasites on ornamentation. If so, carotenoid-based signals could reveal information about an individual's oxidative balance and resistance to oxidative stress: only high-quality individuals, with more antioxidants and a prime antioxidant system, could afford to allocate more carotenoids to pigment their ornaments (von Schantz et al., 2009; Alonso Alvarez et al., 2008; Pike et al., 2007b).

As a model species, we used the red grouse *Lagopus lagopus scoticus* Lath., a game bird that displays bright red supra-orbital combs. The size of combs is testosterone-dependent (Moss et al., 1979; Mougeot et al., 2005a) and plays key roles in intra- and inter-sexual selection: males with bigger combs are more aggressive and achieve greater mating and breeding success (e.g. Bart and Earnst, 1999; Redpath et al., 2006b). The red colour of grouse combs is due to carotenoid pigmentation of the integument (Mougeot et al., 2007a; Egeland et al., 1993; Hollett et al., 1984; Johnsgard, 1983). Bigger combs need more carotenoids for colouration (greater skin surface to pigment) and are often, but not always, redder (F.M. and J.M.-P., unpublished data). The main macro-parasite of red grouse is the nematode *Trichostrongylus tenuis* Eberth., which has well known negative effects on host condition, energy balance, breeding and survival (Delahay et al., 1995; Hudson, 1986a; Mougeot et al., 2005c; Redpath et al., 2006a). *Trichostrongylus tenuis* also limits comb size (Mougeot et al., 2005b; Mougeot et al., 2009a), plasma carotenoid levels and comb redness (Martinez-Padilla et al., 2007; Mougeot et al., 2007b) in male red grouse.

We manipulated *T. tenuis* parasites in free-living males using an anthelminthic drug effective at purging grouse of their *T. tenuis* worms (Hudson, 1986b; Mougeot et al., 2005b). We sampled control and dosed males before treatment and again after 13 days. We investigated treatment effects on ornamentation (comb area and comb redness), plasma carotenoid concentration and oxidative balance. The latter was evaluated by measuring the total antioxidant status of plasma (TAS; an index of circulating antioxidant defences) and plasma concentrations of malondialdehyde (MDA; a measure of oxidative damage). TAS measures the capacity of plasma samples to inhibit a redox reaction induced by free radicals (Prior and Cao, 1999). MDA is formed when lipid hydro-peroxides break down, a process (lipid peroxidation) caused by oxidative stress (Mougeot et al., 2009a; Romero et al., 1998).

We predicted that parasite reduction would enhance ornamentation (comb area and comb redness) and increase carotenoids and TAS (antioxidant defences), while reducing MDA (oxidative damage). At the individual level, we expected treatment effects to be proportional to the changes in *T. tenuis* abundance (greater size effect of parasite purging in males that initially had more *T. tenuis* worms). We also evaluated the relative importance of changes in plasma carotenoids, TAS or MDA in explaining individual changes in comb size and colouration.

MATERIALS AND METHODS

Experimental protocol

We conducted the experiment on Catterick Moor, north Yorkshire (UK) in 2006. In September, we caught 29 male red grouse, by dazzling and netting them at night (Hudson, 1986b). Upon first capture, each male was individually tagged with a radio-collar (TW3-necklace radio-tags, Biotrack, Wareham, Dorset, UK) to facilitate recapture. We measured the maximum length and width of a flattened comb (with a ruler, nearest 1 mm) to calculate comb area (length×width) as an index of ornament size (Mougeot et al., 2005a). We took a digital photograph of the left comb against a standard grey card, and a blood sample from the brachial vein. Plasma was fractionated from blood by centrifugation (2 min at 8000 ***g***), then frozen in liquid nitrogen within 5 min of collection. Plasma samples were taken to the laboratory and stored at –80°C until analysed for assaying carotenoids, TAS and MDA (see below). We kept birds overnight in individual holding pens to collect a faecal sample, used to estimate *T. tenuis* abundance (see below). Prior to release early the next morning, males were given one of two treatments. Control males were given a 1 ml oral dose of water, while dosed males were given a 1 ml of Levamisole Hydrochloride (Nilverm Gold, Schering-Plough Animal Health, Welwyn Garden City, UK) to purge them of their *T. tenuis* nematodes (Hudson, 1986b).

We recaptured males 13±4 days later and remeasured comb area, took another digital photograph of the left comb, a blood sample and separated and kept plasma as described above. We collected another faecal sample, removed the radio-tag and released each bird. We held the necessary Home Office licenses for conducting the procedures described in this work (License PPL80/1437).

The Levamisol drug is usually cleared by red grouse within 10 days (F.M., unpublished data), so we recaptured and remeasured males after this period of 10 days. In chickens, a dose of Levamisol three-times greater than the one we used was undetectable in plasma after three days (El-Kholy and Kemppainen, 2005). In other species, Levamisol was found to have an immunomodulatory effect [e.g. mammals and fish (Quershi et al., 2000; Findlay et al., 2000)]. For this reason, we sampled after the drug was cleared, and we sampled more treated males than control males, in order to test whether the changes caused by treatment were proportional to the number of parasites removed. If greater reductions in MDA and greater increases in TAS and carotenoids are found in birds that had more worms initially, then we can be confident of detecting an effect of the parasite removal, and not merely a possible confounding additive effect of the same dose of Levimasol drug received by treated males.

Parasite abundance estimates

Trichostrongylus tenuis is a significant parasite of red grouse. This gut nematode has a direct life style and no alternative hosts within the same habitat. Eggs laid by adult worms are voided onto the moor in faecal droppings, where they develop into infectious larvae and are ingested by grouse when feeding on heather *Calluna vulgaris* (Hudson, 1986b). We estimated *T. tenuis* abundance using faecal egg concentration from samples collected fresh from live males. Faecal samples were stored at 5°C to inhibit parasite egg development, and processed within two weeks of collection to ensure

reliable estimates (Seivwright et al., 2004). For each sample, a sub-sample of *ca*. 0.2 g of faeces was diluted in 5 ml of saline water and mixed thoroughly. A sub-sample of this solution was placed in a MacMaster slide under a ×40 microscope in order to count the eggs. Faecal egg concentrations (eggs per gram of faecal material) provide reliable estimates of *T. tenuis* abundance (number of worms per grouse), which we calculated using equations provided in Seivwright et al. (Seivwright et al., 2004).

Plasma carotenoid concentration

Plasma carotenoids were quantified using spectrophotometry. Firstly, 60 µl of plasma was diluted in acetone (1:10 dilution). The mixture was vortexed and centrifuged at 7000 ***g*** for 10 min to precipitate flocculent proteins. The supernatant was examined in a Shimadzu UV-1603 spectrophotometer (Kyoto, Japan) and we determined the optical density at 446 nm, the wavelength of maximal absorbance for lutein in acetone (Mínguez-Mosquera, 1993). Plasma carotenoid concentration (µg ml^{-1}) was calculated using a standard curve of lutein (Sigma Chemicals, St Louis, MO, USA). Repeatability was determined on a sub-sample measured twice (R=0.99, N=20, P<0.001).

Comb colour measurements

We measured the redness of grouse combs using digital photographs. High resolution lateral pictures of the head showing the flattened left comb were taken before and after experimental manipulations. Pictures were taken at a standard distance (40 cm) using the camera flash. For each photograph, we placed the same grey reference next to the comb (see Martinez-Padilla et al., 2007; Mougeot et al., 2007b). We analysed images with Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA). For each picture, we recorded the average components red, green and blue (RGB system) within an area of the comb and grey reference. We calculated the 'redness' of the colour of comb and grey reference by subtracting the green and blue scores from the red score. By using this relative difference, we were essentially measuring comb hue, while also capturing variation in saturation [a high value means redder and more saturated combs (Mougeot et al., 2007b)]. RGB comb values, standardised for the RGB value of the grey reference, are hereafter referred as to 'comb redness'.

Total antioxidant status (TAS)

TAS concentration of plasma was assessed using an automated spectrophotometer (A25-Autoanalyzer; Biosystems SA, Barcelona, Spain), and samples were prepared using a commercial kit (Randox Laboratories Ltd, Crumlin, UK). Plasma samples were incubated for 15 s with a chromogen composed of metmyoglobin and ABTS [2,2-azino-di-(3-ethylbenzthiazoline sulphonate)]. Hydrogen peroxide (H_2O_2) was then added and the sample was incubated for 195 s. H_2O_2 addition induces the production of the radical cation ABTS, which generates a blue–green colour. Sample absorbance was measured at 600 nm before and after H_2O_2 addition; thus, determining the change in colour. Antioxidants in the plasma sample cause suppression of this colour change to a degree that is proportional to their concentration. Results are given as mmol l^{-1} of plasma. Repeatability was determined on a sub-sample measured twice (R=0.92, N=30, P<0.001).

Lipid peroxidation assays

Plasma concentrations of MDA were calculated by high-performance liquid chromatography (HPLC) using fluorescence detection as described by Agarwal and Chase (Agarwal and Chase, 2002) with some modifications. All chemicals were HPLC grade, and chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore, Watford, UK). Assays were carried out in 2 ml capacity screw-top micro-centrifuge tubes. To a 15 µl aliquot of sample or standard (1,1,3,3-tetraethoxypropane, TEP; see below) 15 µl butylated hydroxytoluene solution (0.05% w/v in 95% ethanol), 120 µl phosphoric acid solution (0.44 mol l^{-1}) and 30 µl thiobarbituric acid solution (42 mmol l^{-1}) were added. Samples were capped, vortex mixed for 5 s, then heated at 100°C for 1 h on a dry bath incubator to allow formation of MDA–TBA adducts. Samples were then cooled on ice for 5 min, before 75 µl *n*-butanol was added and tubes were vortex mixed for 30 s. Tubes were then centrifuged at 15,338 ***g*** and 4°C for 3 min, before a 50 µl aliquot of the upper (*n*-butanol) phase was collected and transferred into an HPLC vial for analysis. Samples (10 µl) were injected into a Dionex HPLC system (Dionex Corporation, California, CA, USA) fitted with a 5 µm ODS guard column and a Hewlett-Packard Hypersil 5 µm ODS 100×4.6 mm column (Dionex Corporation, CA, USA) maintained at 37°C in a thermostatted column compartment (TCC-100; Dionex). The mobile phase was methanol-buffer (40:60, v/v), the buffer being a 50 mmol l^{-1} anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5 mol l^{-1} 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 (3 µmol l^{-1} in 40% ethanol) serially diluted using 40% ethanol. Repeatability TEP standards assayed in duplicate showed high repeatability (R=0.99, N=11, P<0.0001).

Statistical analyses

We used SAS 8.01 (SAS Institute, Cary, NC, USA). We tested whether changes over time in study parameters differed according to treatment using General Linear Mixed Models that included time (before *vs* after treatment), treatment (control *vs* dosed males) and the time×treatment interaction as fixed effects, and 'individual' as a random effect to account for repeated measures before and after treatment. We used mixed models because of the nature of the data set (repeated measures and unbalanced sample sizes). We did not always have enough plasma to measure all of the physiological parameters (carotenoid, TAS and MDA) at a given sampling time, and we did not obtain a faecal sample for measuring *T. tenuis* abundance on some occasions. Therefore, not all of the parameters could be measured for all individuals on each sampling (before and after treatment), so sample size varied depending on analyses (unbalanced data set). Dependent variables were fitted to models using a normal error distribution and identity link function (comb area, comb redness, CAR, TAS and MDA) or with a negative binomial error distribution and log link function (*T. tenuis* abundance). We calculated individual changes over time in study parameters (Δcomb area, Δcomb redness, ΔCAR, ΔMDA and ΔTAS) as the difference between final and initial values, corrected for the initial values (residuals from a GLM of the difference on the initial values). For correlation analyses, we used Spearman correlations because of our small sample size and of the non-normal distribution of some variable (*T. tenuis* abundance). When evaluating which physiological changes best explained the observed changes in ornamentation (comb area and redness), we used stepwise regressions with a backward model selection, using the AIC criteria for selecting the most parsimonious models [Proc REG (SAS Institute)]. Initial models included changes in plasma carotenoid concentration (ΔCAR), changes in TAS (ΔTAS) and changes in MDA (ΔMDA).

RESULTS

Parasites, oxidative balance and ornamentation before treatment

In unmanipulated males, *T. tenuis* abundance was negatively related to comb redness and TAS but was not significantly related to CAR, MDA or comb area (Table 1). Comb area was negatively related to MDA but was not significantly related to TAS or CAR (Table 1). Comb redness was positively correlated with TAS but was not significantly related to CAR, MDA or comb size. Finally, CAR was not correlated with TAS or MDA, and TAS and MDA were also unrelated (Table 1). Thus, prior to manipulations, males with more *T. tenuis* worms had less TAS and less red combs, and males with bigger combs had lower levels of oxidative damage, as indexed by MDA.

Effect of treatment on *T. tenuis* abundance

Prior to treatment, there was no significant difference between control and dosed males in *T. tenuis* abundance (Genmod procedure, χ^2=1.15, P=0.36, d.f.=21). However, changes over time (before and 13 days after treatment) in *T. tenuis* abundance significantly differed between treatment groups (Fig. 1A; significant time×treatment interaction: $F_{1,19}$=100.5; P<0.001). After treatment, dosed males had no *T. tenuis* worms (N=18), while control males had still, on average, 222 worms (range 0–484; N=6). Thus, the anthelminthic treatment had been effective at purging *T. tenuis* worms.

Effect of treatment on plasma carotenoids, TAS and MDA

Plasma carotenoid concentration, plasma TAS or plasma MDA concentration did not differ significantly between control and dosed males prior to treatment (GLMs: plasma carotenoids: $F_{1,24}$=0.09; P=0.76; TAS: $F_{1,24}$=0.03; P=0.87; MDA=$F_{1,24}$=0.11; P=0.74). Changes over time in plasma carotenoid concentration, plasma TAS or plasma MDA concentration did not differ significantly between control and dosed males (Fig. 1D,E,F; non-significant time× treatment interactions for carotenoids: $F_{1,20}$=1.16; P=0.293; TAS: $F_{1,22}$=0.75; P=0.396; and MDA: $F_{1,21}$=1.33; P=0.261). However, among dosed individuals, changes in plasma carotenoid and changes in TAS were negatively related to changes in parasite abundance (carotenoids: r_s=–0.65; P<0.05; N=10; TAS: r_s=–0.77; P<0.01; N=13). By contrast, changes in MDA concentration were positively related to changes in parasite abundance (r_s=+0.55; P<0.05; N=13). Thus, following parasite purging, a greater reduction in *T. tenuis* parasite abundance was associated with a greater increase in circulating carotenoids and plasma TAS, and a greater reduction in MDA (Fig. 2).

Effect of treatment on comb area and comb colour

Comb area ($F_{1,27}$=1.79; P=0.19) and comb redness ($F_{1,27}$= 0.57; P=0.48) did not differ between control and dosed males prior to treatment. However, changes over time in comb area and in comb redness differed between treatment groups (time×treatment interactions: $F_{1,25}$=7.39; P=0.012 and $F_{1,25}$=4.16; P=0.032, respectively). Comb area and redness tended to decrease in control males but increased in dosed males (Fig. 1B,C). Among dosed males, changes in comb area and comb redness were not significantly related to changes in parasite abundance (Δ comb area: r_s=–0.39; P=0.17; N=14; Δ comb redness: r_s=–0.04; P=0.89; N=14). Changes in comb area and comb redness were also unrelated (r_s=–014; P=0.57; N=19).

We further evaluated which physiological changes best explained the observed changes in ornamentation (comb area and redness). For changes in comb area, the selected model using AIC criteria [Proc REG, (SAS Institute)] only included ΔCAR ($F_{1,18}$=9.35; P<0.01; estimate ± s.e.: 5.67±1.86; model R^2=0.34) but not ΔTAS or ΔMDA (both P>0.15). A greater increase in comb area was only associated with a greater increase in circulating carotenoids. For changes in comb redness, the selected model retained ΔMDA ($F_{1,18}$=8.44; P<0.01; estimate ± s.e.: –17.20±3.14; Fig. 3) and ΔCAR ($F_{1,18}$=5.67; P=0.03; estimate ± s.e.: +2.41±1.01; model R^2=0.49) but not ΔTAS (P>0.15). A greater increase in comb redness was associated with a greater reduction in MDA (Fig. 3) and a greater increase in circulating carotenoids.

DISCUSSION

Parasites and oxidative balance

Our experimental treatment was effective at contrasting *T. tenuis* parasite abundance between groups. Although the treatment was effective, *T. tenuis* abundance was quite low, given that red grouse often host thousands of these parasites and intensities are sometimes as high as 20,000 worms (Hudson, 1986a; Redpath et al., 2006a; Seivwright et al., 2005). Before treatment, we found that comb redness was negatively correlated with *T. tenuis* abundance, consistent with the explanation that parasitism caused carotenoid availability to be limiting. At the treatment group level, we found that parasite reductions led, as expected, to enhanced comb size and carotenoid-based colouration: comb area and redness increased in dosed males but decreased in control males. However, changes in comb size and redness were not proportional to the parasite reduction: males that initially had more worms did not increase comb area or redness more than those with few worms initially.

Treatment had no significant effect on changes in circulating carotenoids, TAS or MDA, although the differences were in the expected directions (tendency for dosed males to increase carotenoids and TAS, and to reduce MDA, with opposite trends in control birds; Fig. 1). However, at the individual level, the relative changes in CAR, TAS and MDA were proportional to the number of *T. tenuis* removed: a greater parasite reduction was associated with a greater reduction in oxidative damage (MDA) and a greater

Table 1. Relationships (Spearman correlations) between study parameters in unmanipulated males (measured before treatment)

	Comb area	Comb redness	CAR*	MDA†	TAS‡
Trichostrongylus tenuis worm abundance	r_s=–0.008, P=0.967	**r_s=–0.540, P=0.008**	r_s=–0.197, P=0.404	r_s=–0.325, P=0.162	**r_s=–0.437, P=0.047**
Comb area		r_s=0.122, P=0.528	r_s=0.114, P=0.586	**r_s=–0.456, P=0.019**	r_s=0.041, P=0.842
Comb redness			r_s=–0.106, P=0.612	r_s=–0.164, P=0.438	r_s=0.274, P=0.175
CAR*				r_s=0.030, P=0.889	r_s=0.204, P=0.328
MDA†					r_s=0.135, P=0.517

Sample size: 23–29 males. Relationships significant at the 0.05 level are highlighted in bold.
*Plasma carotenoid concentration.
†Plasma concentration of malondialdehyde.
‡Total antioxidant status of plasma.

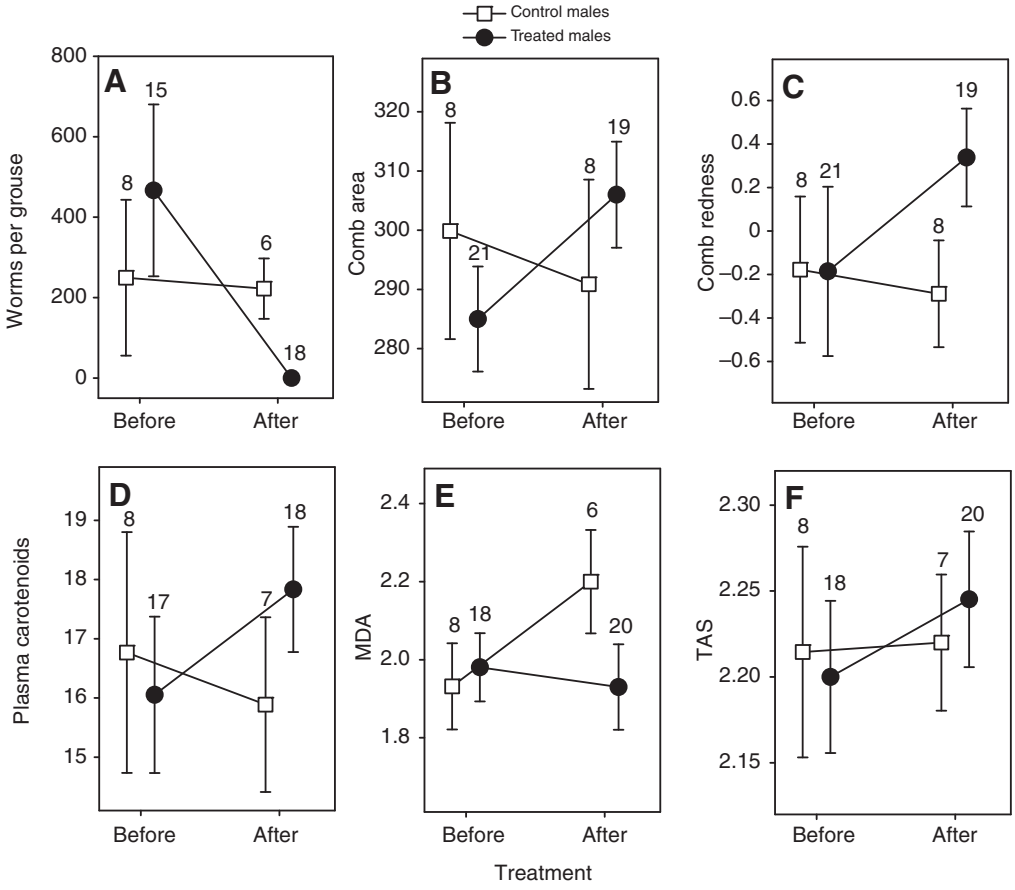

Fig. 1. Mean ± s.e.m. changes over time in (A) *T. tenuis* abundance (worms male^{-1}); (B) comb area (mm^2); (C) comb redness (adjusted *R*-value; see Materials and methods); (D) plasma carotenoid concentration (mg ml^{-1}); (E) plasma concentration of malondialdehyde (MDA; nmol ml^{-1}) and (F) plasma TAS (total antioxidant status; mmol ml^{-1}). White squares: control males; black squares: treated (dosed) males (purged of parasites). Numbers above error bars refer to sample size (number of males).

increase in circulating carotenoids and antioxidant defences (TAS). A possible explanation is that birds that had initially the highest infections showed improved oxidative status after treatment because parasite infection caused oxidative damage proportionally to infection levels. An alternative explanation could be that birds that had initially few *T. tenuis* worms were originally the healthiest and more resistant individuals; because the treatment was effective at purging worms, these males could have shown the greatest increases in MDA and reduction in TAS levels. The first explanation seems most likely, because it is corroborated by previous experiments on red grouse, in which males were challenged with *T. tenuis* parasites: experimental infections reduced circulating carotenoids and TAS, and increased MDA concentration (see Mougeot et al., 2007b; Mougeot et al., 2009a).

Changes in oxidative balance markers within individuals are often not reported in this field of study, with more emphasis typically being put on treatment level effects. CAR, TAS and MDA appeared to respond to small variations in parasite abundance, and the lack of a significant treatment-level effect could have been due to the small sample size (eight control birds) or the overall low *T. tenuis* abundance of study birds. The observation that these physiological parameters responded proportionally to the number of *T. tenuis* removed also argues against a possible confounding (immunostimulatory) effect of the Levamisol drug, but rather supports the hypothesis that *T. tenuis* nematodes limit plasma carotenoid and TAS levels, and causes increased MDA concentration.

A negative relationship between circulating carotenoids and *T. tenuis* abundance has been found in other populations with greater infection levels [range 0–5000 worms (Mougeot et al., 2007b)]. Correlatively, we also found a negative relationship between plasma TAS and *T. tenuis* abundance, consistent with the suggestion that circulating antioxidant defences (as indexed by plasma TAS) were depleted by infection with *T. tenuis* worms. Thus, infection by *T. tenuis* probably limited both circulating carotenoids and antioxidant defences, particularly when parasite abundance was high (Fig. 2). *Trichostrongylus tenuis* can limit carotenoid availability by reducing carotenoid absorption, because of competition with the parasite for absorbed carotenoids, by carotenoid depletion when mounting an immune response to *T. tenuis* or by reducing the ability to metabolise and mobilise carotenoids due to energetic constraints. *Trichostrongylus tenuis* parasites could account for reduced levels of antioxidant activity (TAS) in a number of ways. Firstly, this could be explained by carotenoids being in limited supply, if carotenoids act as antioxidants *in vivo*, a possibility that is currently debated in birds (see below). Secondly, by damaging the gut lining (Shaw, 1988), *T. tenuis* worms might also reduce the absorption of antioxidants other than carotenoids, principally vitamins A and E, or by impacting on host condition might also affect circulating levels of uric acid, contributing to reducing overall antioxidant activity.

Immune system activation produces ROS/RNS to help counter invading pathogens (Hõrak et al., 2007; Romero et al., 1998) but their overproduction can lead to oxidative stress, incurring damage to host tissues, including ornaments, when individuals lack sufficient antioxidant protection (Halliwell et al., 2007; Splettstoesser et al., 2002; von Schantz et al., 1999). Our experimental results are consistent with the idea that *T. tenuis* parasites caused increased levels of oxidative damage (and thus oxidative stress), because removing parasites reduced levels of oxidative damage (MDA) proportionally to the number of worms removed. Oxidative stress

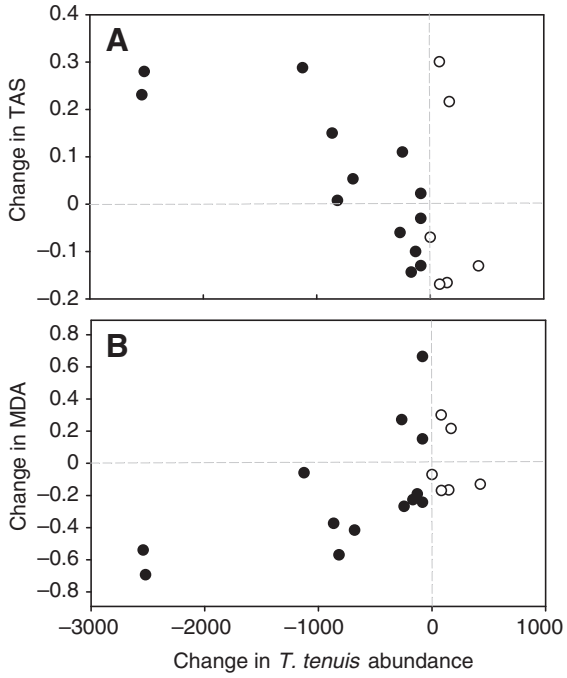

Fig. 2. Relationship between the change in *T. tenuis* worms burdens and changes in (A) plasma TAS (total antioxidant status; mmol ml^{-1}) and (B) plasma concentration of malondialdehyde (MDA; nmol ml^{-1}) in control birds (open circles) and in birds dosed with anthelminthic (black circles).

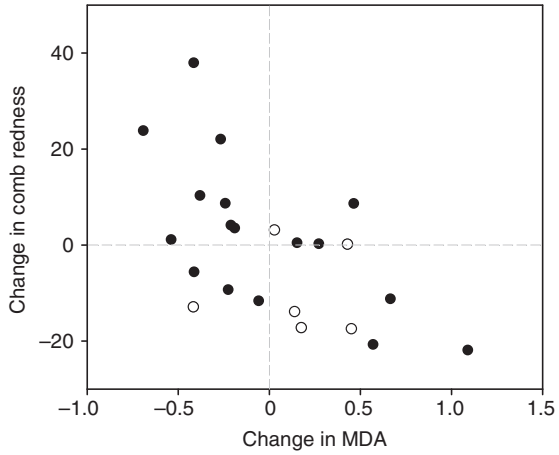

Fig. 3. Relationship between the change in plasma concentration of malondialdehyde (MDA; nmol ml^{-1}) and the change in comb redness (*R*-value) in control males (open circles) and in males dosed with anthelminthic (black circles).

in infected males could have been caused by immune response to *T. tenuis* generating ROS/RNS in combination with a reduction of circulating antioxidant defences. A direct effect of *T. tenuis* parasite on oxidative balance was also supported by parasite challenge experiments, which simultaneously reduced circulating antioxidants (TAS) and increased oxidative damage (MDA) (see Mougeot et al., 2009a).

Oxidative stress as a link between parasites and carotenoid-based ornamentation

When investigating which physiological changes best explained the impact of parasites on changes in ornamentation, we found that a greater increase in comb area was explained by a greater increase in circulating carotenoids, and that a greater increase in comb redness was explained by both a greater reduction in oxidative damage (MDA) and a greater increase in circulating carotenoids. A greater carotenoid-based ornamentation is typically associated with more circulating carotenoids (McGraw, 2006; Mougeot et al., 2009b). Accordingly, greater increases in comb area and redness were both associated with an increase in circulating carotenoids, because bigger and redder combs require more carotenoids for their pigmentation (greater skin surface to pigment and greater pigmentation per surface area, respectively). Most interesting was the tight relationship between the reduction in MDA and the increase in comb colouration after parasite removal. This suggests that carotenoid-based colouration is tightly linked to oxidative balance, i.e. susceptibility to oxidative stress.

Carotenoids have often been hypothesised to be both significant immuno-stimulants and antioxidants in birds (Lozano, 1994; Møller et al., 2000; von Schantz et al., 1999) and therefore carotenoid colouration could be linked to oxidative stress *via* the dual function of carotenoids as pigments and antioxidants. If so, individuals would face an allocation trade-off between carotenoid-based ornamentation and other physiological (antioxidant) functions (Von Schantz et al., 1999; Alonso Alvarez et al., 2008). The immuno-stimulatory properties of carotenoids could be linked to antioxidant defences: mounting an immune response produces ROS/RNS and carotenoids may enhance immune responsiveness by scavenging these molecules (Burton, 1989; Chew et al., 2004). However, recent studies have criticised the assumption that carotenoids are significant antioxidants *in vivo* in birds (Costantini et al., 2007; Costantini and Møller, 2008; Hartley and Kennedy, 2004), although further research is required (see Perez-Rodriguez, 2009). For instance, carotenoid supplementation does not enhance the ability to cope with oxidative stress in nestlings (Costantini et al., 2007). Immune system activation depletes circulating carotenoids but has not always been found to be associated with increased oxidative damage (Alonso-Alvarez et al., 2004; Perez-Rodriguez et al., 2008). However, the latter studies used spectrophotometric measurements to quantify levels of MDA, a technique that is susceptible to interfering chromogens (e.g. Li and Chow, 1994). There is a need for further studies that explore the effects of experimental immune activation on markers of oxidative damage (e.g. MDA; F_2-isoprostanes) using more sensitive and specific HPLC approaches.

From a chemistry perspective, the radical scavenging function of substances, such as carotenoids, does not only depend on their oxidative potential but also on their anti-reductant potential: carotenoids are better defined as antiradical, an action that can take place either accepting or donating electrons (Martinez et al., 2008). ROS/RNS are produced as by-products of oxidation/reduction reactions (Dowling et al., 2009), and the processes through which carotenoids can scavenge ROS/RNS are by donating or accepting electrons. In that respect, red carotenoids, such as asthaxanthin, have a high electron acceptor capacity whereas vitamin E has a high electron donor capacity (Martinez et al., 2008). Therefore, allocating red carotenoid pigments, such as asthaxanthin, to ornaments (a main pigment in red grouse combs) (Mougeot et al., 2007a) could be to the detriment of ROS/RNS scavenging *via* a reduced anti-reductant potential, rather than a reduced oxidation potential.

Carotenoid-based signals could also be sensitive to oxidative stress even if carotenoids are minor antioxidants (Bertrand et al., 2006; Hartley et al., 2004; Perez-Rodriguez et al., 2008; Pike et al., 2007b); for instance, if they are themselves sensitive to attacks by ROS/RNS. If so, by displaying a more coloured carotenoid-based ornament, an individual could signal that it prevented its carotenoids from succumbing to free radical attacks and was able to allocate these for ornamental display, thereby revealing lower levels of oxidative stress or a more efficient antioxidant system. Under such a scenario, carotenoids would not need antioxidant properties to signal an individual's oxidative stress status.

In conclusion, we have shown that the impact of a parasite on a carotenoid-based ornament is tightly linked to the effect of parasites on oxidative balance, and in particular on levels of lipid peroxidation, here indexed by MDA. An individual's ability to enhance carotenoid-based ornamentation after parasite removal was proportional to the reduction in oxidative damage. Oxidative stress, which has been implicated in the aetiology of many diseases, could therefore link carotenoid-based ornamentation and disease or parasite resistance (von Schantz et al., 1999). Sexual traits are expected to be highly integrated with physiological quality (Badyaev, 2004) so the complex interactions between parasites, carotenoids, antioxidant defences and oxidative stress might play a role in conferring honesty on carotenoid-based signals. Carotenoid-based signals might inform about individual levels of oxidative stress even if carotenoids are not powerful antioxidants *in vivo*, but more work is needed to elucidate the exact mechanisms.

LIST OF ABBREVIATIONS

CAR	plasma concentration of carotenoids
MDA	plasma concentration of malondialdehyde
ROS/RNS	reactive oxygen and nitrogen species, respectively
TAS	total antioxidant status of plasma

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