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Parasites, testosterone and honest carotenoid-based signalling of health

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Summary

1. Among the commonest sexual signals of birds are the red-yellow traits pigmented by carotenoids, but how they reliably advertise individual quality remain poorly understood. Here we tested the hypothesis that carotenoid-based signalling is enhanced by testosterone but reduced by parasites, and that the dual action of testosterone on ornament expression and parasite resistance ensures reliable signalling.

2. Tetraonid birds such as the red grouse *Lagopus lagopus scoticus* have bright red combs pigmented by carotenoids, which function in intra- and inter-sexual selection. In separate experiments, we manipulated a main nematode parasite, *Trichostrongylus tenuis* (using deparasitation and re-infection) and testosterone (using testosterone or combined Flutamide/ATD treatments) in free-living males and investigated effects on plasma carotenoids and comb colour.

3. In untreated males, comb redness positively correlated with plasma carotenoids, testosterone concentration and condition. Plasma carotenoids and comb redness both negatively correlated with *T. tenuis* abundance.

4. Plasma carotenoids decreased in response to a challenge from *T. tenuis*, but increased when parasites were reduced. Testosterone enhanced comb redness, but tended to deplete plasma carotenoids. Combined Flutamide and ATD treatment had no significant effects on comb colour or plasma carotenoids, indicating that testosterone effects might be direct.

5. Our experiments show contrasted effects of testosterone and nematode parasites on carotenoid-based ornamentation. Testosterone and parasites have well documented interactions in the study model. These, together with the opposite effects that testosterone and parasites have on carotenoid availability and use, would shape optimal levels of signalling, depending on individual quality, and might ensure reliable signalling.

6. Carotenoid-based and testosterone-dependent traits have rarely been linked. Our study provides such a connection and shows that investigating how parasites, testosterone and carotenoids interact helps in the understanding of the evolution and maintenance of honest carotenoid-based signals of health.

Key-words: ATD, Flutamide, honest signalling, red grouse *Lagopus lagopus scoticus*, sexual selection, *Trichostrongylus tenuis* nematode

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Introduction

The evolution of brightly coloured plumage and ornaments fascinates biologists. A central issue lies in understanding the maintenance of these colourful traits as honest signals of quality (Zahavi & Zahavi 1997). In birds, brightly coloured ornaments often advertise better condition, health or ability to resist parasites (e.g. Hamilton & Zuk 1982; Andersson 1994). Plumage colouration produced by feather structure and pigmentation by carotenoids has received particular attention (e.g. Shawkey & Hill 2005; Hill & McGraw 2006), while fleshy ornaments remain less studied. The colour of integuments or beaks has been shown to change rapidly (e.g. Bortolotti, Fernie & Smits 2003; Faivre *et al.* 2003) and can also provide a useful indicator of current health.

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Carotenoids determine the bright red-yellows of many ornaments, and are among the most familiar targets of female choice (Hill 2002; Hill & McGraw 2006). There is growing interest in understanding the proximate and ultimate causes of variability in carotenoid-based displays (Olson & Owens 1998, 2005; Hill & McGraw 2006), with particular emphasis on sexually selected traits (Andersson 1994; Hill & McGraw 2006) and on the mechanisms constraining or enhancing carotenoid allocation to ornaments (McGraw 2006). Because vertebrates cannot synthesize carotenoids, diet can limit ornament expression (Olson & Owens 1998; Hill, Inouye & Montgomerie 2002; Hill & McGraw 2006). Carotenoids also have important antioxidant and immunostimulant properties (Møller *et al.* 2000; Blount *et al.* 2003; Faivre *et al.* 2003), so individuals can allocate available carotenoids to their ornaments or towards self-maintenance and immune function (von Schantz *et al.* 1999; McGraw & Ardia 2003). According to the parasite-mediated sexual selection hypothesis (Hamilton & Zuk 1982), sexual ornaments, and in particular carotenoid-based ornaments (Lozano 1994), should be particularly sensitive to parasite infections. However, to date, experimental evidence that parasites or diseases negatively influence carotenoid-based signals remains limited (e.g. coccidian parasites: Brawner, Hill & Sundermann 2000; Horak *et al.* 2004; hemoparasites: Horak *et al.* 2001; mycoplasmosis: Hill, Farmer & Beck 2004; nematodes: Martinez-Padilla *et al.* 2007).

The development and expression of sexually selected traits is often under the control of sex-steroid hormones, such as testosterone (Folstad & Karter 1992; Andersson 1994; Hillgarth & Wingfield 1997). The allocation of carotenoids to ornaments can also be modulated by testosterone (e.g. Mundinger 1972; Owens & Short 1995; Eens *et al.* 2000; McGraw, Correa & Adkins-Regan 2006). Testosterone may mediate trade-offs between ornamentation and parasite resistance, which could ensure honest signalling (Folstad & Karter 1992; Verhulst, Dieleman & Parmentier 1999; Eens *et al.* 2000; Getty 2002). In that respect, testosterone-dependent, carotenoid-based signals are particularly interesting because carotenoids can be beneficial for self-maintenance and parasite resistance, given their immuno-stimulant properties (Møller *et al.* 2000; McGraw & Ardia 2003; Alonso-Alvarez *et al.* 2004). For instance, if testosterone increases circulating carotenoids (e.g. McGraw *et al.* 2006), this could provide a buffer for its immunosuppressive effects (Blas *et al.* 2006). Alternatively, mobilization of carotenoids to ornaments by testosterone could withdraw circulating carotenoids, to the detriment of their beneficial physiological effects. This could be part of, or exacerbate, the negative effects that elevated testosterone may have on immuno-competence and parasite resistance. Sexual traits are expected to be highly integrated with physiological quality (Badyaev 2004) so the complex interactions between carotenoids, testosterone, parasites and immuno-competence might play a crucial role in conferring honesty on carotenoid signals (von Schantz *et al.* 1999; McGraw & Ardia 2003). However, to date, very few studies have linked testosterone-dependent and carotenoid-based signals (Blas *et al.* 2006).

Tetraonid birds (grouse family), such as the red grouse (*Lagopus lagopus scoticus*), exhibit brightly coloured supra-orbital combs, which are particularly conspicuous sexual signals. Their size is testosterone dependent (Rintamaki *et al.* 2000; Mougeot *et al.* 2004, 2005a) and plays key roles in intra- and inter-sexual selection: males with bigger and redder combs are more aggressive and achieve greater mating and breeding success (e.g. Bart & Earnst 1999; Rintamaki *et al.* 2000; Redpath *et al.* 2006a). The yellow-red colour of grouse combs is due to carotenoid pigmentation (Hollett, Thomas & MacDonald 1984; Egeland, Parker & Liaaenjensen 1993; Mougeot *et al.* 2007). In capercaillie (*Tetrao ureogallo*), the main carotenoids of combs have been identified as astaxanthin, zeaxanthin, adonixanthin and lutein (Egeland *et al.* 1993). Red grouse combs are pigmented by lutein, astaxanthin and other red carotenoids (Mougeot *et al.* 2007).

We investigated the effects of nematode parasites and testosterone on circulating carotenoids and on comb colouration in free-living male red grouse. We tested two hypotheses. The first was that nematode parasites reduce circulating carotenoids thereby limiting the carotenoid pigmentation of ornaments. The main parasite of red grouse is the nematode *Trichostrongylus tenuis*, which causes significant damage to the caecal mucosa (Shaw 1988) and has negative effects on host condition, energy balance, breeding and survival (Hudson 1986; Delahay, Speakman & Moss 1995; Redpath *et al.* 2006b). We expected plasma carotenoid concentration and comb redness to be negatively related to *T. tenuis* abundance. In an experiment, we manipulated *T. tenuis* parasites abundance and investigated effects on circulating carotenoids. We expected the parasite removals to increase plasma carotenoid concentration, and the parasite increases (challenge with infective larvae) to reduce it.

Second, we tested the hypothesis that testosterone enhances the carotenoid pigmentation of grouse combs, as found in a few other birds (reviewed in McGraw 2006). Thus, we expected comb redness and plasma testosterone concentration to be positively correlated. In an experiment, we removed nematode parasites and assigned males to one of three implant treatments: control, testosterone or combined Flutamide and aromatase inhibitor (ATD) treatment. Flutamide and ATD have been used to block the actions of androgens in various passerine birds (e.g. Soma, Sullivan & Wingfield 1999; Hau *et al.* 2000; Canoine & Gwinner 2002; Moore, Walker & Wingfield 2004; Tomaszycki, Banerjee & Adkins-Regan 2006). Flutamide is an androgen receptor antagonist, while ATD blocks the aromatase enzyme and therefore conversion of androgens to estrogens where they can act through estrogen receptors. In red grouse, Flutamide and ATD treatment

increased circulating testosterone but without enhancing testosterone dependent traits, because the direct actions of testosterone were blocked (Mougeot *et al.* 2005a). We expected testosterone implants to increase plasma carotenoid concentration and comb redness. If testosterone directly influences carotenoid-based signalling, we expected the combined Flutamide and ATD treatment to either reduce or not affect carotenoid levels or comb redness. Alternatively, if testosterone indirectly influence carotenoid-based signalling (i.e. via cascading effects of testosterone on other hormones; see Mougeot *et al.* 2005b) then we expected the Flutamide and ATD to have effects on similar to those of the testosterone treatment.

Methods

GENERAL METHODS

Study species

The red grouse is a medium-sized Tetraonid bird that lives on the heather *Calluna vulgaris* moorlands of the UK. This species is territorial and mainly monogamous. Males establish territories in autumn, and defend these until spring. Pairing starts in the autumn, when females associate with males to obtain breeding territories, and this continues throughout the winter until spring. Breeding take places between March and August (see Hudson 1986 for more details).

Catching, tagging and measuring wild red grouse

We caught wild male red grouse by lamping and netting them at night (Hudson 1986). Each was classified as young (hatched that summer) or old (more than 1-year-old) from plumage traits. Birds were individually ringed and fitted with a radio collar with a unique frequency (TW3 necklace radio tags, Biotrack) to facilitate relocation and recapture in the field. For each male, we measured the maximum length and height of flattened combs with a ruler (nearest 1 mm) and calculated comb area (comb width × height) as a measure of ornament size (see Mougeot & Redpath 2004; Mougeot *et al.* 2005a). We scored the amount of pectoral muscles as a measure of male condition, using a standard method previously used in red grouse studies. This condition score is based on the 'plumpness' of pectoral muscles and ranges from 1 ('skeletal') to 4 ('fat' or 'plump'; see Mougeot *et al.* 2004). We also weighed each bird (with a Pesola spring balance, to the nearest 5 g; male weight averaged 743 ± 51 g (SD), $n = 412$) and measured straight wing length with a ruler (nearest 1 mm) as a measure of size and to calculate the condition index of body mass corrected for size (see Mougeot, Evans & Redpath 2005c).

Experiment 1: parasite manipulations

We conducted this first experiment in autumn 2003 on Edinglassie estate, a grouse moor located in northeast Scotland. We caught 49 males and randomly assigned them to one of two treatments: dosed with anthelminthic (nematode parasite removal) or challenged with parasite larvae (parasite challenge).

Upon first capture (7–15 October), we measured comb size and condition, took a blood sample, and collected a faecal sample for parasite count. Treatment were given after collecting faecal samples to avoid any effect on parasite egg counts (see below), prior to releasing males into the wild. Anthelminthic treated males (or dosed males) were given a 1 mL oral dose of the anthelminthic Levamisole hydrochloride (Nilverm Gold™, Schering-Plough Animal Health, Welwyn Garden City, UK), a treatment effective at removing adult *T. tenuis* nematodes from grouse (see Hudson 1986; Mougeot *et al.* 2004, 2005c). Challenged males were given a 1–2 mL oral dose of distilled water containing *c.* 3000 *T. tenuis* infective larvae previously cultivated in the laboratory (see below).

We re-captured males 28 ± 6 days after treatment (3–12 November). Time between capture and re-capture did not differ significantly between treatment groups (GLM; $F_{1,32} = 0{\cdot}05$, $P > 0{\cdot}50$). Upon recapture, we re-measured condition, weight, took a blood sample and collected a faecal sample. Sample size decreased during the course of the experiment because some males died or could not be re-located. We did not measure comb colour in this experiment.

Experiment 2: hormone manipulations

We conducted this second experiment on three sites in northeast Scotland in autumn 2000 (Edinglassie, Invermark and Invercauld estates) and on one site in northern England in autumn 2002 (Catterick moor, North Yorkshire). We caught 151 male grouse on these sites (90 in 2000; 61 in 2002).

Upon first capture (4 September to 16 October), we measured comb size, weight, wing length and condition, took a digital photograph of the right comb for measuring colour (see below), took a blood sample, and collected a faecal sample. Males were randomly assigned to one of three treatments: control (C-males; 29 males in 2000 and 20 males in 2002); treated with testosterone (T-males; 30 males in 2000 and 23 in 2002); or treated with Flutamide and ATD (FA-males; 31 males in 2000 and 18 in 2002). All birds were implanted with two silastic tubes (each one 20 mm long, 0·62 mm inner and 0·95 mm outer diameter) sealed with silastic glue at both ends. C-males were given two empty implants. T-males were given two implants filled with testosterone proprionate. FA males were given one implant filled with Flutamide (α,α,α-trifluoro-2-methyl-4-nitro-*m*-proprionotoluidide) and another filled with ATD (1,4,6-androstatriene-3,17-dione; Sigma Aldrich, Poole, Dorset). Implants were inserted between skin and pectoral muscles on the flank under local anaesthesia. We previously determined the length of the tubing during trials on captive grouse so that implants would

last for 2–3 months (S. Redpath and F. Mougeot, unpublished data). Before release, we took a blood sample, collected a faecal sample to estimate *T. tenuis* abundance, and orally dosed all males with 1 mL of anthelmintic (Levamisole hydrochloride) to remove their *T. tenuis* worms (Hudson 1986). This allowed standardizing males for possible confounding effects of nematode parasites.

We re-captured males 38 ± 8 days after treatment (18 October to 4 December). Time between capture and re-capture did not differ significantly between treatment groups (GLM; $F_{2,132} = 0{\cdot}17$, $P = 0{\cdot}84$). Upon recapture, we re-measured condition, weight, took a photograph of the right comb and a blood sample. Sample size decreased during the course of the experiment because some males died or could not be re-located. Digital photographs were taken only in autumn 2000. We did not get enough plasma to do both testosterone and carotenoid assays using the same samples, so we used the plasma samples collected in autumn 2000 ($n = 99$) for testosterone assays and those collected in autumn 2002 ($n = 96$) for carotenoid assays.

Additional data on untreated males

To establish the natural relationship between plasma carotenoid concentration and comb redness, we collected additional data on 36 males on Edinglassie estate (northeast Scotland), 16 October to 1 November 2005. We also measured comb size, condition, weight, wing length and collected faecal samples for parasite counts from these males. When analyzing relationships between study parameters in untreated males, we used information from these males, as well as that from males in Experiments 1 and 2 prior to treatments.

SPECIFIC METHODS

Parasite counts and culture of infective larvae for challenges

Trichostrongylus tenuis is a significant parasite of red grouse. This nematode inhabits the caeca of grouse and has a direct life style and no alternative hosts within the same habitat (Hudson 1986; Delahay *et al.* 1995). Eggs laid by adult worms are voided onto the moor in caecal droppings, produced once a day, early morning. Eggs develop into infective larvae and are ingested by grouse when feeding on heather (Hudson 1986).

We estimated *T. tenuis* abundance using faecal egg concentration. After measuring and processing birds, we kept them overnight in individual pens to collect fresh faecal samples produced by grouse early morning. Samples were taken to the laboratory, stored in a cold room at a constant temperature of 5 °C to inhibit parasite egg development, and processed within 2 weeks of collection to ensure reliable parasite abundance estimates (Seivwright *et al.* 2004). For each bird, a subsample of *c.* 0·2 g of faeces was diluted in 5 mL of saline water and mixed thoroughly. A subsample of this solution was placed in a MacMaster slide under a ×40 microscope in order to count eggs. Faecal egg concentrations (eggs per gram) provided reliable estimates of number of worms per grouse (see Seivwright *et al.* 2004). We used the remains of faecal samples collected on first capture to cultivate infective larvae for the parasite challenges, given 1 month later. Details on the methods for cultivating, storing and counting infective larvae for parasite challenges are given in Wilson & Wilson (1978) and Shaw (1988).

Blood sampling

We collected 0·5–1 mL of blood from a sample of males in each experiment by taking a pinprick sample from the brachial vein. Plasma samples were obtained by centrifuging blood for 10 min at 3000 g and were stored at –80 °C until analysed. Plasma samples were used either for testosterone assays or for carotenoid assays.

Plasma carotenoid concentration

Carotenoids were quantified by diluting 60 μL of plasma in acetone (1 : 10 dilution). The mixture was vortexed and centrifuged at 7000 g for 10 min to precipitate the flocculent proteins. The supernatant was examined in a Shimadzu UV-1603 spectrophotometer and we determined the optical density at 446 nm, the wavelength of maximal absorbance for lutein (Mínguez-Mosquera 1993). Lutein is the commonest circulating carotenoid in birds (Hill & McGraw 2006) and is present in red grouse combs (Mougeot *et al.* 2007). Reflectance at this wave length has been considered as a reliable index of total carotenoids (Blount *et al.* 2003; McGraw 2006). Plasma carotenoid concentration (μg/mL) was calculated using a standard curve of lutein (Sigma Chemicals).

Testosterone assays

Plasma testosterone concentrations were measured using a direct double antibody radioimmunoassay. Duplicate 20 μL plasma samples were assayed. The standards, serially diluted in charcoal-stripped chicken serum, were assayed in triplicate. Both unknown samples and standards were heated to 80 °C for 2 min to denature binding proteins. The primary antibody (8680-1419 Biogenesis, Poole) was used at a dilution of 1 : 3500, and the tracer was [1,2,6,7-3H] testosterone (Amersham Pharmacia Biotech, Bucks). After 24-h incubation, the second antibody (donkey anti-rabbit) was added, and bound and free hormones were separated after a further 24 h by centrifugation at 3500 g. The sensitivity of the assay was 0·06 ng/mL, with intra- and inter-assay coefficients of variance of 8·2% and 12·4%, respectively. Cross-reactivity with other steroids was less than 1%.

Comb colour measurements

We measured the redness of grouse combs using digital photographs (e.g. Villafuerte & Negro 1998; van Oort & Dawson 2005). High resolution lateral pictures of the head showing a flattened comb were taken before and after experimental manipulations. Pictures were taken a standard distance (40 cm) using the camera flash. For each photograph, we placed the same grey reference next to the comb, which provided a colour reference balanced in the red, green and blue that we used for standardizing all comb colour measurements (see Villafuerte & Negro 1998).

We analysed images with Adobe Photoshop 7·0. For each picture, we recorded the average components red, green and blue (RGB system) within an area of the comb and reference. RGB measures give equal scores to each component colour when sampling white (high scores) or black (low scores) colours. The hue of a colour is determined by the relative difference in component colours (red, green or blue). For instance, bright and highly saturated red colours score high in the red component relative to either the green or blue, whereas colours that are less red in hue have a larger green and/or blue component, relative to the red component. We calculated the 'redness' of the colour of comb and grey reference by subtracting the green and blue scores from the red score (van Oort & Dawson 2005). By using this relative difference, we were essentially measuring hue, while also capturing variation in saturation (a high value means redder and more saturated combs). RGB comb values, hereafter referred as to 'comb redness', predicted well redness measures obtained with a reflectance spectrometer (see Appendix).

STATISTICAL ANALYSES

We used SAS 8.01 for all analyses. In untreated males, we tested for associations between variables of interest using Generalized Linear Models (GLMs; type III analyses). We first tested whether variation in a variable of interest was explained by site, date (i.e. sampling date) or age, and kept these variables as fixed effects in subsequent models if significant ($P < 0·05$ level). We tested for non-linear relationships by log-transforming explanatory variables or by including a quadratic term.

Dependent variables were fitted to GLMs using the following error distributions and link functions: body mass (log-transformed), testosterone and carotenoid concentrations (log-transformed), and comb redness: normal error distribution and identity link function; counts of *T. tenuis* parasite eggs: Poisson error distribution and log-link function; condition score: binomial error distribution and logit link function.

Condition score was included as an explanatory variable in GLMs either as a continuous variable or as a class variable, to test whether study parameters varied with condition continuously or if they differed above or below a certain condition threshold.

To test for treatment effects, we investigated whether changes over time in study parameters differed between treatment groups, by including Time (i.e. measures before *vs* after treatment), Treatment and the Time × Treatment interaction as explanatory effects. Generalized Linear Mixed Models (or GLMM; Glimmix SAS 2001) included 'individual' as a random effect, in order to account for the repeated measures on individual males. For Experiment 2, which was conducted on several sites, we also included the variable 'site' as a random effect in our models. For analysing treatment effects on condition index (body mass relative to size), we used body mass as the dependent variable with wing length, as an index of body size, as a covariate (see Darlington & Smulders 2001). For parasite data analyses, the dependent variable was the number of eggs counted, with the mass ($\log_e + 1$) of faecal sample used for the count included as an offset in the models to analyse differences in egg concentrations (SAS 2001). For condition score analyses, score was considered the binomial numerator and the maximum possible score (5) as the binomial denominator (see Crawley 1993). For standardizing comb colour measurements, we included the RGB value of the grey reference as a covariate in all models (all $P < 0·05$).

All tests are two tailed and all data in text are given as means ± SD.

Results

PLASMA CAROTENOIDS, PARASITES AND CONDITION

In untreated males, variation in plasma carotenoid concentration was explained by site (GLM: $F_{2,115} = 28·15$; $P < 0·001$) and sampling date (linear increase during autumn; date effect: $F_{1,115} = 5·18$; $P < 0·05$; quadratic term not significant: $F_{1,115} = 0·39$; $P = 0·53$), but was not explained by age after date and site ($F_{1,115} = 2·23$; $P = 0·14$; $R^2 = 0·34$). After controlling for site and date, variation in plasma carotenoid concentration was explained by *T. tenuis* abundance ($F_{1,111} = 8·90$; $P < 0·01$; $R^2 = 0·40$) and pectoral muscle score (as a regressor: $F_{1,116} = 4·38$; $P < 0·05$; $R^2 = 0·38$), but not condition index (body mass corrected for size: $F_{1,125} = 0·46$; $P = 0·49$; $R^2 = 0·34$). Pectoral muscle score included as a class explanatory variable did not significantly explain variation in plasma carotenoid levels ($F_{3,114} = 1·47$; $P = 0·23$), indicating that carotenoid concentration varied along a condition continuum. Males with more circulating carotenoids had lower pectoral muscle scores and fewer worms (Fig. 1a,c). The negative relationships between plasma carotenoid concentration and parasites or condition score were consistent between sites (non-significant site × parasite and site × condition score interactions). When controlling for *T. tenuis* abundance, plasma carotenoid concentration was still negatively related to condition score (as a regressor; $F_{1,110} = 5·13$; $P < 0·05$; $R^2 = 0·42$). Similarly, when controlling for

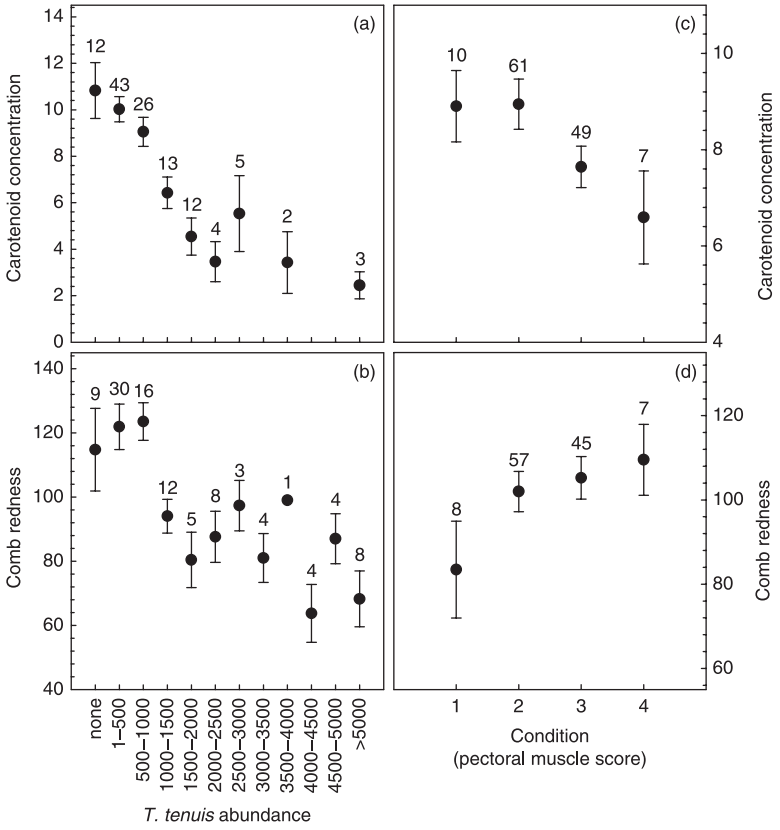

Fig. 1. Variation in plasma carotenoid concentration (in mg/mL) and comb redness according to (a,b) *T. tenuis* abundance (number of worms per grouse) and (c,d) condition (score of pectoral muscles; 1 = 'skeletal'; 4 = 'fat' or 'plump'). Error bars represent standard deviations.

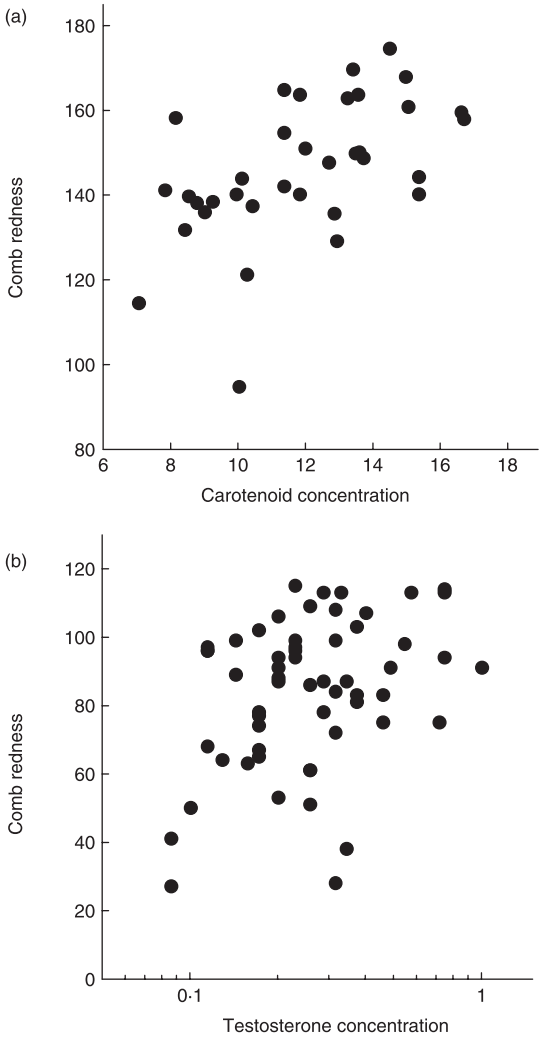

Fig. 2. Relationship between comb redness and (a) plasma carotenoid concentration (in mg/mL) and (b) plasma testosterone concentration (in ng/mL) in untreated male red grouse.

condition score, plasma carotenoid concentration negatively correlated with parasite abundance ($F_{1,110}$ = 8·90; $P < 0·01$; $R^2 = 0·42$).

PLASMA CAROTENOIDS AND COMB REDNESS

We investigated the relationship between comb redness and plasma carotenoids using the data from untreated males in Edinglassie 2005. Variation in comb redness was not explained by age (GLM: $F_{1,32} = 0·00$; $P = 0·97$) or date (date: $F_{1,32} = 0·08$; $P = 0·78$; date^2: $F_{1,32} = 0·07$; $P = 0·80$; $R^2 = 0·02$). However, comb redness positively correlated with (log-transformed) plasma carotenoid concentration (GLM: $F_{1,33} = 19·62$; $P < 0·001$; Fig. 2a; $R^2 = 0·29$).

COMB REDNESS, PARASITES AND CONDITION

In untreated males, variation in comb redness was significantly explained by site (GLM: $F_{3,112} = 36·01$; $P < 0·001$), but was not explained by sampling date (linear date effect: $F_{1,110} = 0·63$; $P = 0·43$; quadratic term: $F_{1,110} = 1·07$; $P = 0·30$) or by age after site ($F_{1,111} = 0·14$; $P = 0·71$; $R^2 = 0·71$). After controlling for site, comb redness variation was explained by *T. tenuis* abundance ($F_{1,98} = 4·39$; $P < 0·05$; $R^2 = 0·75$) and pectoral muscle score ($F_{1,111} = 7·69$; $P < 0·01$; $R^2 = 0·73$), but not by condition index (body mass corrected for size: $F_{1,110}$ = 0·79; $P = 0·38$; $R^2 = 0·72$). Pectoral muscle score included as a class explanatory variable also significantly explained variation in comb redness ($F_{3,114} = 1·47$; $P = 0·23$; $R^2 = 0·74$). Post-hoc tests indicated that males with a condition score of 1 (worse condition) had combs less red that those with a higher condition score (pairwise comparisons, all $P < 0·01$), while comb redness did not differ between males with a condition score of 2, 3 or 4 (all $P < 0·38$; see Fig. 1d). Thus, males with less red combs had a poor condition (pectoral muscle score < 2) and more worms (Fig. 1b,d). The significant relationships between comb redness and parasites or condition score were consistent between sites (non-significant site × parasite and site × condition score interactions). When controlling for condition score (as a class variable), the relationship between comb redness and parasite abundance became marginally significant ($F_{1,96} = 3·33$; $P = 0·071$; $R^2 = 0·77$). However, when controlling for *T. tenuis* abundance, the comb redness variation was still significantly explained by condition score ($F_{3,96} = 5·03$; $P < 0·01$; $R^2 = 0·77$).

EFFECTS OF PARASITE TREATMENTS ON PARASITES, CONDITION AND PLASMA CAROTENOIDS

Parasite treatments were effective at contrasting *T. tenuis* abundance between groups (GLMM with individual as a random effect: Time: $F_{1,25\cdot1} = 9\cdot91$; $P < 0\cdot01$; Treatment: $F_{1,31\cdot9} = 12\cdot91$; $P < 0\cdot01$; Time × Treatment: $F_{1,25\cdot1} = 15\cdot40$; $P < 0\cdot001$). In dosed birds, parasite abundance significantly decreased over time (slope estimate: $-4\cdot281 \pm 0\cdot579$; $P < 0\cdot001$; reduction from 591 to 0 *T. tenuis* worms per grouse; $n = 25$ and 17, respectively), while in challenged birds it significantly increased over time (slope estimate: $0\cdot997 \pm 0\cdot417$; $P = 0\cdot023$; increase from 488 to 972 worms per grouse; $n = 23$ and 16, respectively).

Changes in body mass of individual males before and after treatment did not differ between treatment groups (GLMM: Time: $F_{1,33} = 0\cdot01$; $P = 0\cdot92$; Treatment: $F_{1,33} = 0\cdot07$; $P = 0\cdot79$; Time × Treatment: $F_{1,33} = 0\cdot25$; $P = 0\cdot62$). Similarly, changes in pectoral muscle score over time did not differ between dosed and challenged males (GLMM: Time: $F_{1,80} = 0\cdot41$; $P = 0\cdot52$; Treatment: $F_{1,44} = 0\cdot19$; $P = 0\cdot67$; Time × Treatment: $F_{1,80} = 0\cdot31$; $P = 0\cdot58$). We had thus no evidence that the parasite manipulations influenced these measures of condition during the course of the experiment.

Parasite manipulations significantly affected changes in plasma carotenoid concentration (GLMM: Time: $F_{1,19} = 0\cdot05$; $P = 0\cdot83$; Treatment: $F_{1,19} = 0\cdot00$; $P = 0\cdot97$; Time × Treatment: $F_{1,19} = 4\cdot92$; $P < 0\cdot05$). In dosed males, plasma carotenoid concentration tended to increase over time (slope estimate: $0\cdot267 \pm 0\cdot195$; $P = 0\cdot09$), while it significantly decreased in males challenged with parasites (slope estimate: $-0\cdot326 \pm 0\cdot183$; $P = 0\cdot039$; Fig. 3). We did not measure comb colour in this experiment.

COMB REDNESS AND TESTOSTERONE

In untreated males, comb redness positively correlated with (log-transformed) plasma testosterone concentration (GLM: Site: $F_{1,49} = 1\cdot05$; $P = 0\cdot36$; Testosterone: $F_{1,49} = 4\cdot15$; $P < 0\cdot05$; $R^2 = 0\cdot15$; Fig. 2b).

EFFECTS OF HORMONE TREATMENTS ON TESTOSTERONE CONCENTRATION AND CONDITION

We first evaluated the effects of the hormone treatments on plasma testosterone levels. Changes over time in testosterone concentration differed between treatment groups (GLMM: Time: $F_{1,29} = 92\cdot18$; $P < 0\cdot001$; Treatment: $F_{2,29} = 22\cdot84$; $P < 0\cdot001$; Time × Treatment: $F_{2,29} = 20\cdot44$; $P < 0\cdot001$; Fig. 4a). Testosterone concentration increased significantly more in testosterone implanted males (T-males) than in control males (C-males) (Time × Treatment: $F_{1,18} = 75\cdot19$; $P < 0\cdot001$). Testosterone concentration also increased more in males implanted with Flutamide and ATD (FA-males) than in C-males (Time × Treatment: $F_{1,22} = 21\cdot67$; $P < 0\cdot001$; Fig. 4a), and this increase in testosterone concentration was similar to that observed in T-males (Fig. 4a).

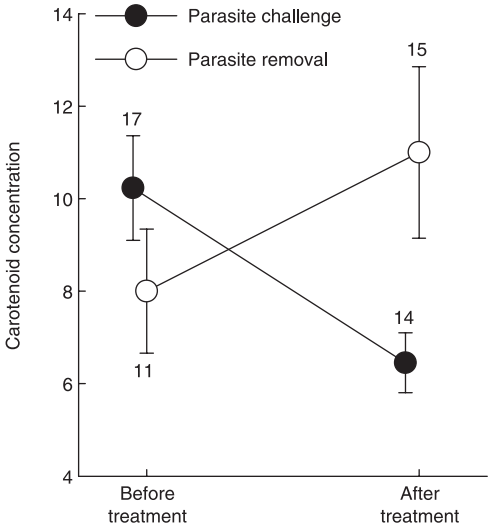

Fig. 3. Effect of parasite manipulations on changes over time in mean ± SE plasma carotenoid concentration (in mg/mL).

Hormone treatments had no significant effect on changes over time in body mass (GLMM: Time: $F_{1,119} = 3\cdot28$; $P = 0\cdot07$; Treatment: $F_{2,119} = 0\cdot69$; $P = 0\cdot51$; Time × Treatment: $F_{2,119} = 0\cdot24$; $P = 0\cdot79$) or condition score (GLMM: Time: $F_{1,91\cdot9} = 1\cdot61$; $P = 0\cdot21$; Treatment: $F_{2,141} = 1\cdot99$; $P = 0\cdot13$; Time × Treatment: $F_{2,91\cdot9} = 0\cdot41$; $P = 0\cdot52$). Males tended to gain weight between capture (726 ± 77 g, $n = 150$) and recapture (738 ± 42, $n = 122$), similarly so in T-, C-, and FA-males.

EFFECTS OF HORMONE TREATMENTS ON CAROTENOID CONCENTRATION AND COMB REDNESS

Plasma carotenoid concentration tended to increase between initial capture and recapture, possibly as a result of deparasitation. Hormone treatments had no significant effect on changes over time in carotenoid concentration (Time: $F_{1,36} = 2\cdot95$; $P = 0\cdot09$; Treatment: $F_{2,36} = 0\cdot76$; $P = 0\cdot48$; Time × Treatment: $F_{2,36} = 0\cdot92$; $P = 0\cdot41$; Fig. 4b). Changes were not as marked in T-males than in C- males or FA-males (Fig. 4b), but differences were not statistically significant (T-males *vs* C-males: Time × Treatment interaction: $F_{1,24} = 0\cdot67$; $P = 0\cdot42$; T-males *vs* FA-males: Time × Treatment interaction: $F_{1,23} = 0\cdot16$; $P = 0\cdot69$).

Hormone treatments affected changes in comb redness (Time: $F_{1,50} = 1\cdot34$; $P = 0\cdot25$; Treatment: $F_{2,50} = 12\cdot58$; $P < 0\cdot0001$; Time × Treatment: $F_{2,50} = 11\cdot11$; $P < 0\cdot001$; Fig. 4c). Comb redness increased significantly more in T-males than in C-males (Time × Treatment interaction: $F_{1,31} = 20\cdot43$; $P < 0\cdot0001$; Fig. 4c), but changes in comb redness did not differ between FA-males and C-males (Time × Treatment interaction: $F_{1,34} = 0\cdot05$; $P = 0\cdot82$; Fig. 4c).

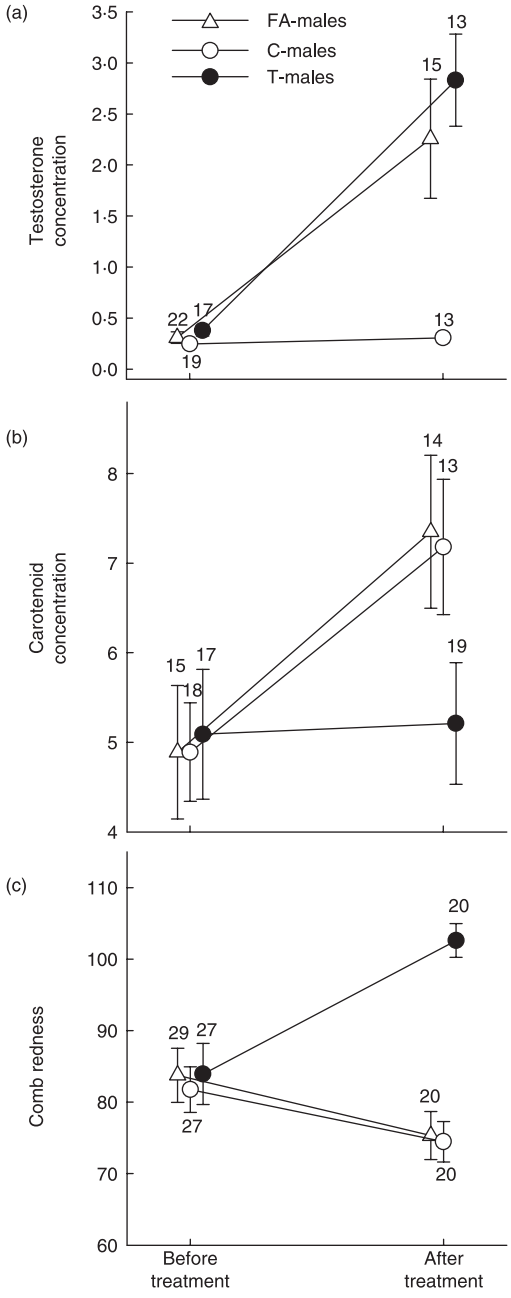

Fig. 4. Effects of hormone treatments on changes in mean ± SE (a) plasma testosterone concentration (in ng/mL); (b) plasma carotenoid concentration (in mg/mL) and (c) comb redness (see Methods).

Discussion

In agreement with our predictions, we found contrasted effects of nematode parasites and testosterone on carotenoid-based signals in free-living male red grouse. Infection by *T. tenuis* nematodes reduced circulating levels of plasma carotenoids and influenced comb redness, while testosterone enhanced the size and carotenoid pigmentation of ornaments. Below we discuss these findings and their implications for our understanding of parasite mediated sexual selection and how carotenoid-based signals evolved and are maintained as honest indicators of health.

PARASITES AND CAROTENOID-BASED SIGNALLING

Carotenoid-based signals should be particularly sensitive to parasite infections (Lozano 1994; Hill & McGraw 2006). Our correlative results showed that both plasma carotenoids and comb redness decreased with increasing *T. tenuis* abundance in male red grouse. Through our experiments we were able to manipulate parasite intensity and demonstrated a direct causal role for *T. tenuis* nematodes in reducing circulating carotenoids. Reducing *T. tenuis* abundance by administrating an oral dose of anthelminthic was effective as birds had no detectable worms 1 month after treatment and increased plasma carotenoids. In contrast, the challenge with infective larvae increased *T. tenuis* abundance by *c.* 500 worms and decreased circulating carotenoids. In previous experiments (Mougeot *et al.* 2005b; Seivwright *et al.* 2005; Mougeot, Redpath & Piertney 2006), about a third of challenged larvae successfully developed into mature worms, as found in this study.

In untreated birds, abundance ranged from 0 to more than 5000 worms per grouse, while the parasites manipulations contrasted abundance by *c.* 1000 worms. Between 0 and 1000 worms, the correlative data suggest little impact of *T. tenuis* on circulating carotenoids or comb redness but the experimental results showed that the nematodes can significantly reduce circulating carotenoids. This suggested that carotenoid-based signalling might be sensitive to small differences in parasite abundance, and that over the natural range of parasite infections (abundance can be as high as several 10 000s worms; Hudson 1986), *T. tenuis* worms could greatly impact on the carotenoid pigmentation of combs. Levels of carotenoids in plasma predict integument colouration in at least six bird species (e.g. McGraw 2006), and we found the same for red grouse. We did not measure comb redness in the parasite manipulation experiments, but we would expect the treatments to have impacted on comb colour, because comb redness correlates positively with circulating carotenoids (Fig. 2a) and negatively with *T. tenuis* abundance (Fig. 1b). This was confirmed by a recent experiment conducted on red grouse (Martinez-Padilla *et al.* 2007).

Nematodes can reduce circulating carotenoids and impact on ornamental colouration in several, non-exclusive, ways. First, like coccidian parasites, nematodes might disrupt carotenoid absorption (Allen 1987). *Trichostrongylus tenuis* is known to cause significant damages to the caecal mucosa (Shaw 1988; Delahay *et al.* 1995), and might thereby disrupt carotenoid absorption. Second, worms and hosts might compete for carotenoid in the caeca, with parasites withdrawing carotenoids that could otherwise be available for absorption by hosts. Third, after ingestion through diet, carotenoids are incorporated into micelles and enter in the mucosal cells. The metabolic route followed by carotenoids from acquisition through diet

up to deposition in ornaments can involve several metabolic transformations, and is most likely to be energetically costly (Hill 2002). Parasites might negatively impact on host condition and energetics (Wilson & Wilson 1978; Delahay *et al.* 1995) and could thereby influence this metabolic route. Fourth, worms might reduce lipoprotein levels that transport and mobilize carotenoids (McGraw *et al.* 2006). Finally, intestinal parasites might increase the demand for carotenoids for immunological functions (Møller *et al.* 2000). Grouse infected by *T. tenuis* mount an immune response to the infection (Wilson & Wilson 1978; Mougeot *et al.* 2004), which would produce free radicals and increase oxidative stress (Ahmad 1995). Carotenoids may lose their pigmentary properties when attacked by free radicals produced during immune response (Hartley & Kennedy 2004), which could lead to a decrease in carotenoid availability for ornamentation (Lozano 1994). More detailed work is required to distinguish between these different possible actions of nematodes on carotenoid availability and use.

CONDITION AND CAROTENOID-BASED SIGNALLING

Ornament expression is expected to be condition-dependent (Andersson 1994; Cotton, Fowler & Pomiankowski 2004) and several studies have found condition-dependence in carotenoid-based signals, like plumage (Hill & McGraw 2006) or bill (Peters *et al.* 2004) colour. This might be because animals cannot synthesize carotenoids and only good foragers ingest enough carotenoids to show bright colour (e.g. Hill & McGraw 2006). Nematode parasites negatively impact on condition (Gulland 1995) and in red grouse this usually occur at abundance greater than 2000–3000 worms (Hudson 1986). Raising an immune response is often costly (Sheldon & Verhulst 1996), so condition-dependent ornamentation might be mediated by parasite effects and parasite defence needs. In male red grouse, both comb size and comb redness are condition-dependent (Mougeot *et al.* 2004, 2007; this study). Comb were less red below a certain threshold condition (condition score < 2), with males in worst condition (lowest amount of pectoral muscles) showing less red combs. Comb redness also appeared more related to condition than to parasites, since *T. tenuis* abundance explained less well variation in comb redness after controlling for condition score. In another study where parasite abundance was low (< 500 worms) and did not impact on condition, comb redness positively correlated with both condition and *T. tenuis* abundance (Mougeot *et al.* 2007). This also suggested that variation in comb redness might relate to condition, or the ability of males to cope with *T. tenuis*, rather than *T. tenuis* abundance itself when hosts have few parasites.

Our parasite manipulations affected plasma carotenoid levels, but without significantly changing condition. This might be because the duration of the experiments was too short to detect such effects or because *T. tenuis* abundance was low overall. This also suggested that parasites can have short-term effects on carotenoid availability and physiology not directly linked to their effects on host body condition or body reserves like fat or pectoral muscles.

Unexpectedly, the relationship between condition score and plasma carotenoids was negative. If males in good condition have redder combs, and redder combs are associated with more circulating carotenoids, then, a better condition should also be associated with more circulating carotenoids. A possible explanation is that individuals in good condition did not need to maintain high levels of circulating carotenoids. They might have allocated more carotenoids to ornamentation or stored more carotenoids in other compartments (Negro *et al.* 2001) thereby withdrawing them from the bloodstream. In any case, individuals in prime condition allocated more carotenoids to their ornaments.

TESTOSTERONE AND CAROTENOID-BASED SIGNALLING

The effect of sex-steroids on carotenoid based-signals remains poorly understood, although there is evidence that sex steroids, and testosterone in particular, can influence carotenoid-based signals (e.g. Eens *et al.* 2000; Stoehr & Hill 2001; McGraw *et al.* 2006). In zebra finches *Taeniopygia guttata*, for example, testosterone treatment increased beak colour, by up-regulating the production of lipoprotein that binds and transports carotenoids, thereby enhancing the pigmentation of ornaments (McGraw *et al.* 2006).

In red grouse, we found that comb redness positively correlated with plasma testosterone concentration and with comb size, which is testosterone dependent (Mougeot *et al.* 2004, 2005a). Testosterone implants enhanced comb redness, supporting a role for testosterone in carotenoid-based signalling. Interestingly, the positive effect of testosterone on comb redness found here was not accompanied by an increase in plasma carotenoids, as found in other studies (Blas *et al.* 2006; McGraw *et al.* 2006). In fact, testosterone-implanted birds tended to have less circulating carotenoids than control males after a month, despite maintaining their high levels of testosterone. Testosterone implants might have initially increased circulating carotenoid levels, by enhancing absorption or transport, but these might have declined afterwards while the increase in comb redness was maintained. This has been observed in red legged-partridge *Alectoris rufa*, with testosterone implants causing a short-term increase in circulating carotenoids followed by a decrease after 10 days (Blas *et al.* 2006). An alternative explanation might be that testosterone implants changed allocation priorities for the available carotenoids rather than causing an increase in carotenoid absorption or transport (McGraw *et al.* 2006). Testosterone-treated males might have been forced to allocate their available

carotenoids to ornaments, to the detriment of maintaining high circulating levels or use for other functions (von Schantz *et al.* 1999).

Using a combined Flutamide and ATD (FA) treatment, we tested whether the actions of testosterone were direct or indirect. In zebra finches, a similar anti-androgen treatment successfully reduced beak colour, by down-regulating lipoprotein production and carotenoid circulation (McGraw *et al.* 2006). FA-males had significantly more testosterone than controls after a month, as observed in similar experiments in other birds (Soma *et al.* 1999; Moore *et al.* 2004). This was presumably a consequence of the treatment blocking a negative feedback and causing increased endogenous testosterone production (see Mougeot *et al.* 2005a). However, the FA treatment failed to change circulating carotenoid levels or comb redness after a month. Elsewhere, we also showed that the FA treatment did not affect comb size, aggressive behaviours (Mougeot *et al.* 2005a), territory size or breeding success (Mougeot *et al.* 2005b), in contrast with the effects of a testosterone treatment (see Moss, Parr & Lambin 1994; Mougeot *et al.* 2005a; Redpath *et al.* 2006a). It is remarkable that, after a month, the FA treatment caused a marked increase in circulating testosterone (similar to that observed in testosterone implanted males) without this being associated with higher or lower plasma carotenoid concentration or enhanced comb redness. This might be because the Flutamide and ATD doses used were not sufficient to fully block the actions of testosterone. It also suggested that the actions of testosterone on ornamental colour were direct rather than indirect, as found in zebra finches (McGraw *et al.* 2006). If the Flutamide and ATD treatment effects had been similar to the testosterone treatment, it would have indicated an indirect effect of testosterone on carotenoid-based signalling (see Mougeot *et al.* 2005b). The elevated testosterone levels observed in both T- and FA-males could have caused an increase in another hormone (for instance, corticosterone), which could have more directly influenced ornament expression, given that the actions of the other hormones would not have been blocked by either the testosterone treatment or FA treatment.

TESTOSTERONE × PARASITE INTERACTIONS AND CAROTENOID-BASED SIGNALLING

According to the immunocompetence handicap hypothesis or ICHH (Folstad & Karter 1992) elevated testosterone enhances the expression of sexual traits but is associated with immuno-suppressive effects and a reduced ability to resist parasite infections. This double-edge sword effect would ensure honest signalling (Folstad & Karter 1992).

Carotenoid-based signals in birds might directly signal male health because carotenoids can act as immune enhancers and antioxidants (they can directly boost the immune system and enhance immunocompetence; Blount *et al.* 2003; McGraw & Ardia 2003; Alonso-Alvarez *et al.* 2004). There is also evidence that immune activation decreases circulating carotenoids and testosterone levels, and reduces carotenoid-based colouration (Faivre *et al.* 2003; Peters *et al.* 2004). Therefore, a trade-off between allocating carotenoids to fight parasites or diseases versus allocating them to ornaments might ensure honest sexual signalling (Lozano 1994; McGraw & Ardia 2003). Diseased and parasitized individuals allocate fewer carotenoids to their ornaments not only because parasites reduce carotenoid availability but also because these individuals need them as antioxidants and for resisting parasites (e.g. Thompson *et al.* 1997; Brawner *et al.* 2000). In red grouse, signal honesty might be reinforced by the testosterone-mediated allocation of carotenoids to ornament expression. Highly parasitized individuals have less carotenoids available for comb pigmentation, a situation which could be exacerbated when testosterone also impairs the ability of individuals to cope with parasites.

Although there is mixed evidence that testosterone is immuno-suppressive in birds (Roberts, Buchanan & Evans 2004), a number of studies have shown that elevated testosterone can impair an individual's ability to cope with current parasites (e.g. Eens *et al.* 2000) or to respond to a standardized parasite challenge (e.g. Duckworth *et al.* 2001). In red grouse, experiments have shown that testosterone reduces cellular immunity (Mougeot *et al.* 2004), and that testosterone and parasites interact in two ways. First, testosterone decreases the ability of males to cope with a standardized challenge with *T. tenuis* infective larvae (Seivwright *et al.* 2005; Mougeot *et al.* 2006) by increasing host susceptibility (Mougeot *et al.* 2005b). Second, high parasite abundance limits the expression of testosterone-dependent comb size (Fox & Hudson 2001; Mougeot *et al.* 2005c). Here we have shown that nematode parasites and testosterone have contrasted effects on carotenoid-based signalling. Males with bigger and redder combs, typically in better condition (Mougeot *et al.* 2007), might be able to afford having elevated testosterone without compromising immune function and to allocate more carotenoids to their combs. The two-way interactions between *T. tenuis* parasites and testosterone, and their contrasted effects on circulating carotenoid levels and use for ornamental colouration would shape optimal levels of carotenoid-based signalling, depending on an individual's condition, parasite abundance and ability to cope with parasites, and might ensure reliable carotenoid-based signalling of health.

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