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Testing the interactive effects of testosterone and parasites on carotenoid-based ornamentation in a wild bird

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Abstract

Testosterone underlies the expression of most secondary sexual traits, playing a key role in sexual selection. However, high levels might be associated with physiological costs, such as immunosuppression. Immunostimulant carotenoids underpin the expression of many red-yellow ornaments, but are regulated by testosterone and constrained by parasites. We manipulated testosterone and nematode burdens in red grouse (*Lagopus lagopus scoticus*) in two populations to tease apart their effects on carotenoid levels, ornament size and colouration in three time-step periods. We found no evidence for interactive effects of testosterone and parasites on ornament size and colouration. We showed that ornament colouration was testosterone-driven. However, parasites decreased comb size with a time delay and testosterone increased carotenoid levels in one of the populations. This suggests that environmental context plays a key role in determining how individuals resolve the trade-off between allocating carotenoids for ornamental coloration or for self-maintenance needs. Our study advocates that adequately testing the mechanisms behind the production or maintenance of secondary sexual characters has to take into account the dynamics of sexual trait expression and their environmental context.

Introduction

Theoretical models of sexual selection suggest that extravagant ornaments and other secondary sexual traits reflect the phenotypic/genetic quality of the bearer (Zahavi, 1975; Hamilton & Zuk, 1982). The honesty of signals is ensured through the costs associated with the production and maintenance of that signal, such that only high-quality individuals can afford to allocate resources from fitness-related functions such as survival or self-maintenance to the expression of the trait (Zahavi, 1975; Getty, 2002; Ketterson *et al.*, 1991; Kokko, 1998; Getty, 2002; Kokko *et al.*, 2002).

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Hormones often mediate trade-offs between investment in sexual signalling versus other demands (Møller *et al.*, 2005; Rubolini *et al.*, 2006; Kurtz *et al.*, 2007). The immunocompetence handicap hypothesis (ICHH; Folstad & Karter, 1992) posits that testosterone would act as a 'double-edged sword' by enhancing the expression of sexual ornaments but suppressing immune defence (Peters, 2000; but see Roberts *et al.*, 2004; Owens-Ashley *et al.*, 2004; Kurtz *et al.*, 2007). This physiological cost would ensure the honesty of the signal, and as such only high-quality individuals could afford the immunosuppressive effect of testosterone and concomitant costs of higher parasite burden (Hamilton & Zuk, 1982). Furthermore, the cost of increasing testosterone or parasites above a certain threshold could increase the likelihood of paying a higher cost for low-quality individuals (see e.g. Mougeot *et al.*, 2006).

There has been much less focus on the extent to which trade-offs may be mediated by processes influencing ornament colouration. Carotenoids might play a key role in mediating the the trade-offs hypothesised by the ICHH, as they can be allocated to ornament pigmentation or to enhance immune responsiveness. Specifically, carotenoids underpin the colouration of many yellow-red ornaments in vertebrates, many of which are targets of female choice during mating (Hill *et al.*, 2002; Hill & McGraw, 2006). Carotenoids are immunostimulants (Blount *et al.*, 2003; McGraw & Ardia, 2003) that cannot be synthesized *de novo* by animals (Olson & Owens, 1998; Hill & McGraw, 2006) despite the fact that there is also some heritable variation in carotenoid-based traits (Fitze *et al.*, 2003). Individuals might allocate available carotenoids to their ornaments or towards immune function and parasite resistance (Lozano, 1994; von Schantz *et al.*, 1999), and the trade-offs resulting from carotenoid use in self-maintenance versus ornamentation might confer honesty to the signal (Faivre *et al.*, 2003). The ICHH does not predict carotenoid priority allocations, but understanding how carotenoids are allocated is essential to understand the, yet unknown, mechanisms acting within the ICHH. In fact, the dual function of carotenoids on ornament development and immune function has led to suggest that carotenoids may buffer testosterone-mediated immunosuppression (Blas *et al.*, 2006; McGraw & Ardia, 2007). Hence, it has been recently proposed that carotenoids may play a pivotal role as a currency in the trade-offs between increased testosterone levels (and subsequently, ornament expression) and self-maintenance (Alonso-Alvarez *et al.*, 2009). Within the ICHH, the relationship between testosterone and carotenoids would mechanistically explain the honesty of the secondary sexual traits: testosterone could only increase carotenoid availability for ornamentation in high-quality individuals, because in low-quality individuals bioavailable carotenoids would have to be allocated to compensate the testosterone-induced immunosuppression.

Both testosterone and parasites affect ornament expression (Mougeot *et al.*, 2007b). Ornament size and colouration can be enhanced by high testosterone titres (Mougeot *et al.*, 2004, 2007b, 2009) but reduced by parasites (e.g. Horak *et al.*, 2004; Mougeot *et al.*, 2007b). In a similar way, testosterone enhances carotenoid availability (Blas *et al.*, 2006; McGraw *et al.*, 2006) by up-regulating the production of lipoprotein that binds and transport them (McGraw & Parker, 2006; McGraw *et al.*, 2006) or by increasing absorption capacity in the intestine (Blas *et al.*, 2006). Intestinal parasites can also affect carotenoid levels in birds (Horak *et al.*, 2001, 2004), and in some cases might damage gut cells and therefore limit carotenoid acquisition (Horak *et al.*, 2001, 2004; Martínez-Padilla *et al.*, 2007). Although the individual effects of testosterone (Blas *et al.*, 2006; Mougeot *et al.*, 2007b) or parasites (Horak *et al.*, 2004; Martínez-Padilla *et al.*, 2007) on ornamentation have been examined widely, the combined, integrated effects of these two factors on ornamentation size, colouration and carotenoid availability remain untested.

Here, we exploit a previous large-scale field manipulation on red grouse (*Lagopus lagopus scoticust* – see Mougeot *et al.*, 2009; Bortolotti *et al.*, 2009) to investigate the interactive effects of testosterone and parasites on carotenoid availability and use for colouration of integument ornaments in a wild bird. We simultaneously manipulated levels of testosterone, and levels of infections by a main intestinal parasite, using a fully factorial design replicated in two populations. The effect of testosterone and parasite treatments was examined over time to separate short from long-term effects. Fleshy carotenoid-pigmented ornaments of other species have been shown to respond rapidly to external or internal factors (Bortolotti *et al.*, 2003; Faivre *et al.*, 2003), so it is expected that testosterone effects on colouration may be immediate. However, parasites might have a delayed effect on ornamentation, depending of their virulence, life cycle or physiological effects (Mougeot *et al.*, 2009). Thus, we also explore the dynamic of changes over time in carotenoid availability and ornament colouration and size in relation to testosterone and parasite manipulations.

We used the red grouse (*Lagopus lagopus scoticus*) as a model system. Red grouse display supra-orbital combs that are pigmented by carotenoids (Mougeot *et al.*, 2007a,b) and displayed during intra- and inter-sexual interactions (Redpath *et al.*, 2006). Previous studies in this species have shown that testosterone increases ornament size and enhances carotenoid-based colouration (Mougeot *et al.*, 2007b), whereas parasitic nematodes constrain comb size (Seivwright *et al.*, 2005) and comb colouration (Martínez-Padilla *et al.*, 2007). However, when circulating levels of carotenoids are measured, the effects of either parasites or testosterone are not consistent (Mougeot *et al.*, 2007b). Furthermore, it has been shown that *Trichostrongylus tenuis* parasites and testosterone interact in red grouse: testosterone increases susceptibility to parasites (Seivwright *et al.*, 2005), whereas parasites limit the expression of testosterone-dependent signals (Fox & Hudson, 2001; Mougeot *et al.*, 2005a). With our factorial experiment, we attempted to tease apart the relative effects of testosterone and parasites on carotenoid bioavailability and ornament size and colouration. We tested the interactive effect of testosterone and parasites on ornamentation and circulating carotenoids over a 17-days time period. We predicted that (1) testosterone- and parasite-free birds will have the biggest and redder combs together with the highest levels of circulating carotenoids; and (2) the effects on ornament size, colouration and levels of carotenoids would be first detected in testosterone-implanted and parasite-free males.

Methods

General methods

The experiment was conducted in two grouse moors in autumn 2006: (according to Bortolotti *et al.*, 2009 and Mougeot *et al.*, 2009) Edinglassie estate (hereafter refereed as to population 1), located in north-east Scotland and Catterick moor (hereafter population 2) in North Yorkshire, England. Population 1 was at the low phase of its cycle (low density), whereas population 2 was nearing its population peak (see Redpath *et al.*, 2006). In September 2006 (first capture or R0), we caught wild male red grouse by lamping and netting them at night (Hudson, 1986). 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. We caught 40 males (20 in each population). All were kept overnight in individual boxes to collect faecal samples for nematode parasite counts (details below). In the morning before releasing males into the wild, all birds were given a 1ml dose of the anthelminthic levamisole hydrochloride (Nilverm Gold-TM; Schering-Plough Animal Health, Welwyn Garden City, UK), a treatment effective at purging grouse from their adult *T. tenuis* nematodes (see Hudson, 1986; Mougeot *et al.*, 2004, 2005b). Experimental manipulations were conducted 15 days later (upon first recapture or R1), to give enough time for the birds to clear the anthelminthic drug (usually within 10 days; see Martínez-Padilla *et al.*, 2007 for more details).

Experimental manipulations

Upon first recapture (R1), we randomly assigned males to one of four treatment groups:

(1) Testosterone-implanted and no parasite challenge (T+ P−)
(2) Empty implants (Inert) and no parasite challenge (T− P−)
(3) Testosterone-implanted and challenge with 5000 *T. tenuis* infective larvae (T+ P+)
(4) Empty implants and challenge with 5000 *T. tenuis* infective larvae (T− P+).

All birds were implanted with two silastic tubes (20 mm long, 1.57 mm inner and 2.41 mm outer diameter each) sealed with glue at both ends. Testosterone control males (T− males) were given two empty implants, and testosterone-treated males (T+ males) were given two implants filled with testosterone (Sigma Aldrich Co Ltd, Poole, Dorset, UK). Implants were inserted between skin and breast 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). For parasite treatments, challenged males (P+ males) were given a 1–2 mL oral dose of distilled water containing *c.* 5000 *T. tenuis* infective larvae previously cultivated in the laboratory (details below). P− males were given 1.5 mL of water.

Males were recaptured at two further time points after treatment (R2 and R3, 10 and 17 days after R1, respectively). At the third recapture (R3), males were humanely killed to collect their caecum and accurately measure *T. tenuis* abundance (see Mougeot *et al.*, 2009). During the course of the study, three birds were predated between R2 and R3: one in the group T+P− and two in the T+P+, and could not be re-sampled at R3. The rest of the birds were all recaptured at all sampling times. Sample size might be lower than the numbers of birds recaptured for carotenoid levels or parasite abundance estimates either because the volume of blood plasma was not enough to assess carotenoid levels or because individuals did not produce the caecal samples used for measuring parasite abundance (see further details below).

Upon each recapture (R1, R2 and R3), we measured the following for each male: (1) ornament size – 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 *et al.*, 2004, 2005a); (2) comb colour (redness); (3) *T. tenuis* burden (Mougeot *et al.*, 2009); (4) testosterone titre (Mougeot *et al.*, 2009) and (5) plasma carotenoid concentration (details below).

Comb colour measurements

Comb redness was measured using a portable spectrophotometer (Minolta CM-2600D; Konica Minolta, Osaka, Japan). We took two measurements of the same comb and bird at each capture, from which we obtained hue, chroma and brightness values. Measurements of hue, chroma and brightness were highly repeatable (all > 0.80 and $P < 0.001$, Lessells & Boag, 1987) and were averaged. Brightness is a measure of the total amount of light reflected by a surface. Hue can be defined as the everyday perception of colour and indicates what wavelengths contribute most to total reflectance, whereas chroma is an index of saturation or spectral purity of that colour. The hue of the combs of male red grouse ranges from pale orange to deep red. The three variables of colour were reduced using a principal components analysis, then extracting orthogonal components to be interpreted as an index of comb redness. The first principal component (PC1) explained 82.8% of comb colour variation (eigenvalue = 2.48) and was positively correlated with all hue (eigenvector = 0.587), chroma (eigenvector = 0.570) and brightness (eigenvector = 0.574). We used PC1 as an index of comb redness, with higher values being associated with redder, brighter combs with a higher spectral purity.

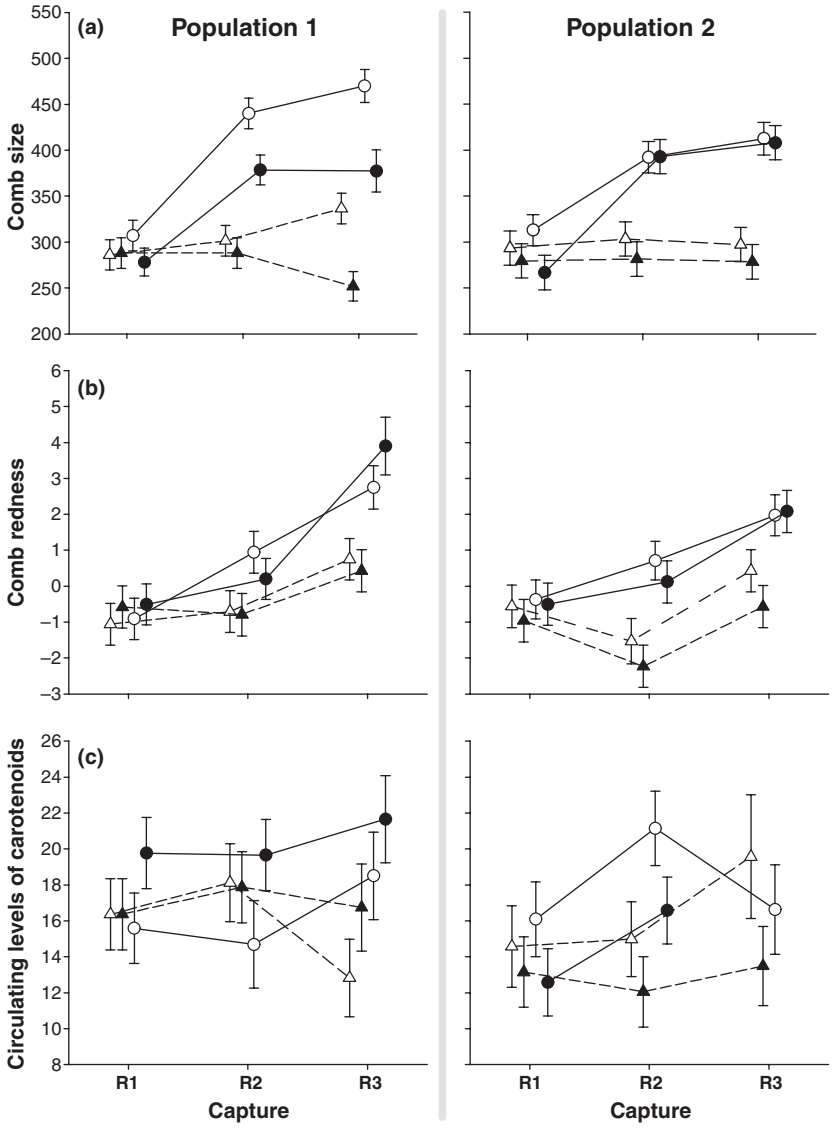

Fig. 1 Changes over time and according to treatments in (mean ± SE) (a) comb size (mm^2); (b) comb redness (adjusted index of redness; see Methods) and (c) circulating levels of carotenoids ($\mu g\ mL^{-1}$) by site from mixed models, which included the bird identity as a random factor. For combined analysis of testosterone and parasite treatment on ornament size in both populations together see Mougeot *et al.*, 2009. Black circles (T+P+), empty circles (T+P−), black triangles (T−P+) and empty triangles (T−P−) denote the different experimental treatments.

Parasite abundance estimates and parasite culture for challenges

Trichostrongylus tenuis is the main nematode parasite of red grouse, infecting the caecum of *c.* 100% of adult birds (Hudson, 1986). It has a direct life style and no alternative hosts within the same habitat (Hudson, 1986). Eggs laid by adult *T. tenuis* worms are voided onto the moor via the host's caecal droppings. Here, they develop into infective larvae and are ingested by grouse when feeding on heather *Calluna vulgaris* (Hudson, 1986). We used the remains of caecal samples collected upon first capture to cultivate *T. tenuis* parasite infective larvae for the subsequent challenges. We followed the methods for cultivation, storing and counting *T. tenuis* larvae described in previous works (Shaw & Moss, 1989; Shaw *et al.*, 1989; Seivwright *et al.*, 2004). Individual doses containing *c.* 5000 *T. tenuis* infective larvae were then extracted and given orally to males (Mougeot *et al.*, 2005a, 2006, 2009; Redpath *et al.*, 2006; Bortolotti *et al.*, 2009). To estimate *T. tenuis* abundance for each male, we used either faecal egg counts (upon R0, R1 and R2, using caecal samples collected from live captured males) or direct worm counts (R3, using caecum collected post-mortem at the end of the experiment). Caecal egg counts provide reliable estimates of worm burdens and were used to calculate worm intensity (see Seivwright *et al.*, 2004). Direct worm counts from the caecum were conducted following Seivwright *et al.* (2004).

Blood sampling

We collected ~ 1 mL of blood from males by intravenous extraction from the brachial vein. Plasma samples were

obtained by centrifuging blood for 10 min at 4500 *g*. The plasma layer was removed and stored at −80 °C prior to testosterone and carotenoid assays.

Testosterone assays

We measured plasma testosterone concentration using a commercially available testosterone enzyme immunoassay (ELISA kit EIA-1559 from DRG Diagnostics, Marburg, Germany; see Mougeot *et al.*, 2009 for further details), which has been developed and validated for determining testosterone levels in small volumes of avian plasma (25 μL). Coefficients of variation intra- and inter-assays were 3.59% and 7.14%, respectively, and the detection limit was 0.2 ng mL^{-1}. Duplicate sample analyses showed that the testosterone assays were highly repeatable ($F_{23,59} = 15.99$, $P < 0.001$; $r = 0.88$).

Plasma carotenoid concentration

A subsample of plasma collected for each capture was used to quantify circulating carotenoids. Carotenoids were quantified by diluting 60 μL of plasma in acetone (1 : 10 dilution). The mixture was vortex mixed, and flocculent proteins were precipitated by centrifugation (7000 *g* for 10 min). The supernatant was examined in a ShimadzuUV-1603 spectrophotometer (Shimadzu, Kyoto, Japan), optical density measurements were taken at 446 nm, the wavelength of maximal absorbance for lutein (Mínguez-Mosquera, 1993), which is the main carotenoid circulated by grouse (Mougeot *et al.*, 2007a). Plasma carotenoid concentration (μg mL^{-1}) was calculated using a standard curve of lutein (Sigma Chemicals). Duplicate sample analyses showed that plasma carotenoids assays were highly repeatable ($F_{19,\ 20} = 602.2$, $P < 0.001$, $r = 0.99$).

Statistical analyses

We used SAS 8.01 (SAS Institute, Inc., Cary, NC, USA) for all analyses. Testosterone levels, carotenoid concentration and comb redness were fitted to models using normal error distribution and identity link function. We used poisson error distribution and log link function for *T. tenuis* parasite egg counts. For parasite estimates from egg counts over time, the dependent variable was the estimated number of worms and time as factor (GLIMMIX models). We refer to Mougeot *et al.* (2009) for the statistical significance of treatments on parasites estimates from worm counts. To assess our treatment effects, we ran four models with testosterone, comb size, comb colouration and levels of circulating carotenoids as dependent variables. For the analyses of treatment effects, we separated treatments into the hormone treatment (HTREAT; empty implants or testosterone implants) and the PTREAT (not challenged or challenged with infective larvae) and looked at their interaction (HTREAT × PTREAT). We tested for differences between treatment groups on individual changes over time (time × treatment interactions) in study parameters. To account for the effect of treatments over time, capture time (time) was included as factor with the three time steps considered altogether. Only when time-by-treatment interaction effects were significant, we explored differences among individual pairs (R1 and R2, or R2 and R3) of recapture times. This way, we could use 20 birds for comparisons over time of HTREAT (for interactions HTREAT × time: 20 control birds against 20 testosterone-implanted birds) or PTREAT (PTREAT × time: 20 control birds against 20 parasite-challenged birds) when analysing their effect on comb colouration and comb size. Consequently, this allowed us to compare the combined effect of testosterone and parasites (HTREAT × PTREAT × time) on individual changes over time between groups of not less than 10 birds per group (T+P+, T+P−, T−P+ and T−P−). When analysing the effect of testosterone independently of parasites, we only used birds that were not challenged with parasites (T+P− and T−P−). Similarly, when analysing the effect of parasites independently of testosterone, we only used birds that were sham-implanted (T−P+ and T−P−). Sample size was reduced for carotenoid levels or parasite estimations, because of missing data (as explained above). Also, because our experiment was replicated in two moors (or 'populations'), the interactions between 'population' and other factors were included as fixed effects. All general linear mixed models (GLMM models) included 'individual' as a random effect, to account for the repeated measures on individual males. In all models, when interactions were not statistically significant, they were removed; therefore, the final models were those that best explained the variance of the dependent variables.

To assess variability within individuals, we calculated individual changes over time in comb size (ΔCsize), comb redness (ΔCred), circulating carotenoids (Δcarot) and testosterone level (ΔT) over the course of the experiment. These changes were calculated as the absolute differences corrected for the initial values. To do so, we included the value of the dependent variable analysed at R1 as a covariate on each model. We explored the relationship between individual changes of (1) comb size and colouration, (2) comb colouration and testosterone, (3) carotenoids and colouration and (4) carotenoids and testosterone in relation to HTREAT, PTREAT and site.

Results

Baseline site differences prior to experimental manipulation

At R0, before purging parasites, *T. tenuis* worm abundance tended to be higher in population 2 (average

866 ± 200 worms per male) than in population 1 (238 ± 181), but this difference was not statistically significant (GLM, $F_{1,41} = 1.88$, $P = 0.178$). Circulating testosterone, comb redness and size did not differ between experimental groups (HTREAT, PTREAT, HTREAT × PTREAT, all $P > 0.130$) or between sites (HTREAT × site, PTREAT × site, HTREAT × PTREAT × site, all $P > 0.625$). Only, levels of circulating carotenoids did vary between the populations ($F_{1,41} = 4.46$, $P = 0.040$) and were significantly lower in population 1 (14.0 ± 3.8 μg mL^{-1}, $n = 10$) than in population 2 (16.6±4.0 μg mL^{-1}, $n = 10$).

Effectiveness of parasite and testosterone treatments

At the time of implanting (R1), *T. tenuis* worms per bird decreased (Bortolotti *et al.*, 2009; Mougeot *et al.*, 2009). Changes over time in parasite abundance between R1 and R2 did not significantly differ between treatment groups (Mougeot *et al.*, 2009). Worm burdens at R3 (measured with direct worm counts, at the end of the experiment) were significantly higher in challenged (P+) than in nonchallenged (P−) males (Mougeot *et al.*, 2009). Between R1 and R3, *T. tenuis* worm burdens increased significantly more in challenged (P+) than in nonchallenged (P−) males (Mougeot *et al.*, 2009). The relative differences in parasite burdens between P− and P+ males were similar between populations ($F_{1,26} = 0.24$, $P = 0.626$), although the number of worms in birds from population 1 was higher than in population 2 at the end of the experiment (population 1, P−: 6.0, P+: 65.7 worms per male; population 2, P−: 58.8, P+: 260.0 worms per male, $F_{1,26} = 3.73$, $P = 0.046$).

Testosterone treatment changed testosterone levels over time (Mougeot *et al.*, 2009) in a similar pattern between sites (HTREAT × times × site $F_{2.16} = 0.85$, $P = 0.447$). Testosterone levels increased between R1 and R2 (Mougeot *et al.*, 2009) independently of site (all $P > 0.250$). However, testosterone levels between R2 and R3 did not differ (Mougeot *et al.*, 2009) at either site ($F_{1,16} = 0.52$, $P = 0.481$), indicating that circulating levels of testosterone remained constant after implanting (R2–R3).

Interactive effects of testosterone and parasites

The combined effect of testosterone and parasite treatment did not change parasite abundance over time (Mougeot *et al.*, 2009) and was independent of site ($F_{2,48} = 0.59$, $P = 0.557$). The combined effect of testosterone and parasites did not change comb size, redness of the comb or circulating levels of carotenoids (HTREAT × PTREAT × time × site, all $P > 0.221$). In all cases, these lack of effects were the same at both sites (HTREAT × PTREAT × time, all $P > 0.269$, Table 1, Fig. 1). We thus had no evidence that testosterone and parasite effects were interactive.

Effect of parasites

Parasite treatment did not change circulating levels of carotenoids or comb redness over time (PTREAT × time, both $P > 0.314$), with no difference between sites (PTREAT × time × site, both $P > 0.706$). However, PTREAT affected changes in comb size over time, depending on site (PTREAT × time × site, $F_{2,70} = 4.28$, $P = 0.017$). Between R1 and R2, parasite challenges did not affect comb size (Mougeot *et al.*, 2009), and this was independent of site ($F_{1,16} = 0.30$, $P = 0.592$). However, parasite challenges influenced comb size between R2 and R3 (Mougeot *et al.*, 2009), depending on site ($F_{1,17} = 10.11$, $P = 0.005$). In population 1, comb size decreased more in challenged birds than nonchallenged males (Table 1, Fig. 1a), but this difference was not found in population 2 (Table 1, Fig. 1a). Therefore, parasite challenges decreased comb size only in population 1, and these changes were only apparent after a 4-week time lag.

Table 1 General Linear Mixed Models (GLMM procedure, SAS, 2001) testing for an effect of testosterone (HTREAT) and parasite (PTREAT) treatments and their interaction on changes over time in carotenoid levels, redness and size of male red grouse combs in each study population. Bold values denote statistically significant effects.

		Population 1							Population 2						
			HTREAT		PTREAT		HTREAT × PTREAT			HTREAT		PTREAT		HTREAT × PTREAT	
Dependent	Recapture	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	d.f.	*F*	*P*	*F*	*P*	*F*	*P*
Carotenoids	R1–R2	1,12	**6.78**	**0.024**	1.33	0.276	0.15	0.707	1,11	0.20	0.663	0.81	0.383	0.43	0.527
Redness	R1–R2	1,15	**15.05**	**< 0.001**	3.71	0.068	0.42	0.664	1,15	**11.95**	**0.003**	1.10	0.309	0.34	0.718
Comb size	R1–R2	1,16	**29.65**	**< 0.001**	0.98	0.338	0.18	0.673	1,16	**45.31**	**< 0.001**	0.10	0.758	2.64	0.124
Carotenoids	R2–R3	1,4	2.81	0.192	0.79	0.440	0.01	0.980	1,7	1.99	0.201	0.19	0.689	0.08	0.921
Redness	R2–R3	1,12	1.52	0.241	1.63	0.226	0.56	0.587	1,14	1.84	0.195	0.21	0.657	0.39	0.682
Comb size	R2–R3	1,13	0.79	0.387	**21.42**	**< 0.001**	1.74	0.210	1,15	0.93	0.350	0.05	0.823	0.42	0.526

Effect of testosterone

Testosterone treatment changed comb size over time (Mougeot *et al.*, 2009), similarly in both sites (HTREAT × time × site, $F_{2,32} = 1.32$, $P = 0.281$). Between R1 and R2, testosterone treatment increased comb size (Mougeot *et al.*, 2009), and this effect was independent of site ($F_{1,17} = 3.03$, $P = 0.091$). Between R2 and R3, comb size did not change in relation to testosterone treatment, with no difference between sites (both $P > 0.303$).

Testosterone treatment affected changes in comb redness (HTREAT × time, $F_{2,31} = 4.65$, $P = 0.017$), similarly across sites (HTREAT × time × site, $F_{2,31} = 0.68$, $P = 0.513$). Between R1 and R2, testosterone treatment increased comb redness ($F_{1,16} = 14.69$, $P = 0.001$, Fig. 1a,b), and this effect was independent of site ($F_{1,16} = 0.63$, $P = 0.438$). Between R2 and R3, comb redness did not change in relation to testosterone treatment and was independent of site (both $P > 0.492$).

Testosterone treatment changed circulating levels of carotenoids over time, but depending on site (HTREAT × time, $F_{2,26} = 2.40$, $P = 0.133$, HTREAT × time × site, $F_{2,26} = 9.42$, $P = 0.005$). Between R1 and R2, testosterone increased circulating carotenoids only in population 1, not in population 2 (HTREAT × time × site: $F_{1,12} = 6.19$, $P = 0.028$; Table 1). Between R2 and R3, carotenoid levels did not change in relation to testosterone treatment and was independent of site (both $P > 0.087$).

Thus, testosterone rapidly (R1–R2) increased comb size and redness, although circulating carotenoids only increased in population 1 (Table 1, Fig. 1c). These changes were still apparent 4 weeks after treatment (R2–R3, all $P > 0.115$).

Individual changes of comb size, redness, testosterone and carotenoids

Between R1 and R2, the relationship between changes in comb size (ΔCsize) and comb redness depended on both HTREAT and site (significant ΔCred × HTREAT × site interaction, $F_{1,38} = 3.85$, $P = 0.037$, ΔCsize as dependent variable; Fig. 2a), but did not vary with PTREAT or a combination of PTREAT and HTREAT (both $P > 0.275$). In population 1, the effect of HTREAT on changes in comb size and redness differed ($F_{1,18} = 5.90$, $P = 0.029$, Fig. 2a). Comb colouration increased with comb size in T+ males (estimate: 48.81 ± 12.71, $F_{1,8} = 14.67$, $P = 0.008$), but not in T− birds ($F_{1,9} = 0.82$, $P = 0.395$). In population 2, HTREAT did not explain the relationship between comb size and colouration ($F_{1,19} = 0.06$, $P = 0.816$; Fig. 2a): changes in comb redness were unrelated to changes in comb size.

Individual changes in comb redness (ΔCred) were related to individual changes in testosterone levels, but the relationship also differed between sites (significant ΔT × HTREAT × site interaction, $F_{1,26} = 7.42$, $P = 0.013$, ΔCred as dependent variable, Fig. 2b). In T+ males, changes in testosterone levels were positively associated with changes in comb redness for population 1 ($F_{1,11} = 11.31$, $P = 0.024$; estimate = 0.416 ± 0.116, $F_{1,6} = 15.66$, $P = 0.010$), but the relationship was opposite in population 2 ($F_{1,14} = 4.16$, $P = 0.048$; estimate =−0.459 ± 0.142, $F_{1,7} = 10.39$, $P = 0.018$). In T− males, changes in comb redness were not related to changes in testosterone levels, in either site (both $P > 0.324$).

The relationships between carotenoid levels and colouration on the one hand, and carotenoid levels and testosterone levels on the other hand, were not mediated by HTREAT (both $P > 0.407$), PTREAT (both $P > 0.136$) or a combination of both treatments (both $P > 0.313$; Fig. 2c). Between R2 and R3, none of the relationships described earlier were significant (all $P > 0.425$).

Discussion

We found that testosterone and parasites acted at different time scales on ornament size (*sensu* Mougeot *et al.*, 2009) and colouration. The effect of testosterone on comb size was faster than that of parasites, and the short-term (i.e. within 3 weeks) effects of both factors were not interactive (Table 1). Interestingly, we found that the effects on comb colouration, but not on comb size, were site-dependent. These results were also supported at the individual level, as the relationships between changes in testosterone and changes in carotenoid-based ornamentation also differed between sites. Our results suggest that the trade-offs associated with the ICHH might differ depending on environmental circumstances and that ornament size and colouration might reveal different aspects of individual quality.

Testosterone and parasite interaction

Previous experiments in red grouse have shown that testosterone manipulation may increase nematode burdens, but with time lag of a year (Mougeot *et al.*, 2005c; Seivwright *et al.*, 2005). The mechanism appears physiological (increased susceptibility) rather than behavioural (increasing exposure: Mougeot *et al.*, 2005c), but the exact physiological mechanism remains unknown. Therefore, our results agree with the idea that the effect of testosterone on parasite infection is not immediate, and whatever the mechanisms acting to resolve the trade-offs between ornamentation and immunity at one point in time, they might have a delayed effect, at least in red grouse. Unfortunately, we can not explore the effects that the parasite and testosterone challenge may have had a year later, but it would be expected that testosterone- and parasite-treated birds would end up with the higher numbers of parasites (Seivwright *et al.*, 2005).

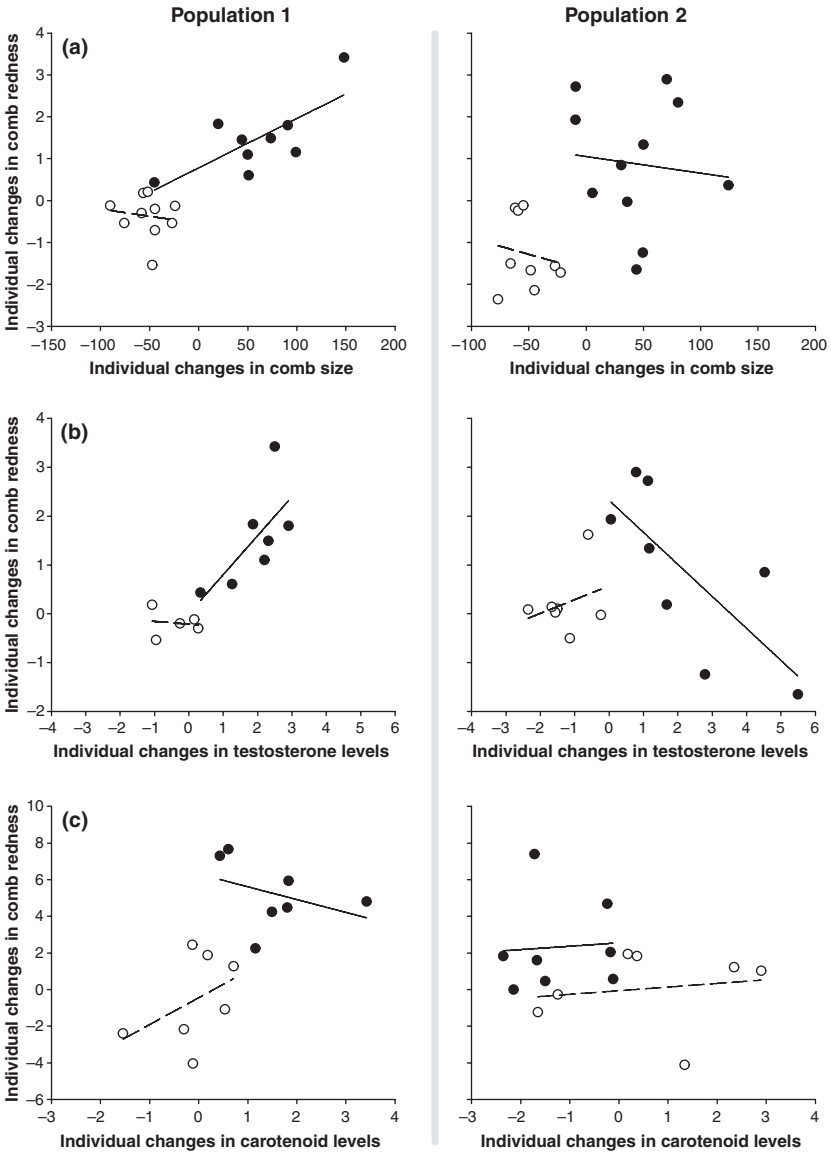

Fig. 2 Relationships between individual changes over time (between R1 and R2) in comb redness (adjusted index of redness; see Methods) and (a) comb size (mm^2), (b) testosterone levels (ng mL^{-1}) and (c) carotenoid levels (μg mL^{-1}). Open dots and dashed lines: sham-implanted birds (T−); black dots and continuous lines: testosterone-treated birds (T+). Because parasite treatment had no effect on these relationships, we pooled challenged (P+) and non-challenged (P−) individuals (see methods and statistics for more details).

We expected that an interactive effect of our experimental treatments would lead to testosterone-implanted and parasite-free males having the bigger and more coloured ornaments. However, we found little support for this interactive effect on ornament size and colouration, at least on a short-term basis. Our results highlight that ornament expression, at least in red grouse, is more testosterone-driven than parasite-driven on a short-term basis. However, as pointed out before, the interactive effect might be delayed: birds treated with testosterone in a given autumn have more parasites the following one (Mougeot *et al.*, 2005c; Seivwright *et al.*, 2005), but they also still have bigger combs (Mougeot *et al.*, 2005b), despite the fact that implants were active only for 3 months. We cannot fully disentangle the mechanisms behind this lack of effect but they do suggest that at least two different physiological pathways might be involved with ornament pigmentation, considering that both factors influence ornament display independently. Our results have important implications regarding the honesty of sexual signals when intrinsic (hormones) and extrinsic (parasites) factors are involved, at least over a short timescale. Ornament size might primarily reveal information about the hormonal status of the individual (i.e. aggressiveness, dominance), whereas comb colouration, mediated by the availability of carotenoids, might rather reflect the physiological constraints the individual is experiencing. Considering our population-dependent effects of hormones on ornamentation, future studies should

consider that the interactive, or lack of, effect of testosterone and parasites could differ between different environmental contexts. We nevertheless have to be cautious when interpreting the lack of interactive effects of testosterone and parasites between populations, because of sample size limitations.

We also predicted that the effects on ornament size, colouration and levels of carotenoids would be first detected in testosterone-implanted and parasite-free males. However, testosterone increased the redness and size of combs, but parasites only affected comb size, and with a time lag in one population. Our results support the idea that both parasites and testosterone influence the expression of secondary sexual traits, as suggested by the ICHH, but the impact of testosterone is quicker than that of parasites. The time lag between parasite challenge and the impact of challenges on comb size can be explained by the time needed for the larvae to develop in adult worms, which is *c.* 14 days (Shaw *et al.*, 1989; Delahay *et al.*, 1995). We suggest, therefore, that the different factors involved in the ICHH may act at different temporal scales, and that these may, in turn, depend of the type of parasite involved.

Parasites and ornamentation

Despite the fact that whilst parasites decreased comb size (Mougeot *et al.*, 2009), this effect was site-dependent. We challenged birds with infective *T. tenuis* larvae (L3 stage of development) that subsequently develop into adult, egg producing worms. This is the stage that causes most damage to gut walls in red grouse (Shaw *et al.*, 1989; Delahay *et al.*, 1995). The relative increase in worm burdens between R1 and R3 was higher for population 1 (from 6.0 to 65.7 worms per male) than population 2 (from 58.8 to 260.0 worms per male), despite the fact that worm abundance did not reach very high numbers (described up to 30.000 worms, see Hudson, 1986). Thus, this greater relative increase in worm burdens could explain why parasites decreased comb size in population 1 but not in population 2.

We also found that parasite treatment did not influence comb colouration or levels of circulating carotenoids, unlike in previous experiments (Mougeot *et al.*, 2007b). Methodological differences may explain the different results. Parasite challenges in red grouse reduced comb colour, but the time elapsed between parasite challenge and recapture was longer (28 days, see Mougeot *et al.*, 2007b) than in this study (R1–R3 was 17 days), suggesting a longer time for parasites to affect host physiology and comb colouration.

Site-dependent effect of testosterone on ornamentation

Experimentally increased testosterone levels caused the expected rapid increases in comb size and redness. Interestingly, testosterone-implants caused a short-term increase in circulating carotenoids in one site (population 1), but not in the other, despite the fact that testosterone increased the carotenoid-based coloration of combs in both sites. In addition, the effect of testosterone on ornamentation remained constant until the end of the experiment. This suggests that the effect of testosterone on ornamentation (comb size and colouration) reaches a ceiling where carotenoid levels and ornamentation no longer change, probably because they have reached a certain threshold above which individuals cannot further increase their carotenoid absorption or circulation (Alonso-Alvarez *et al.*, 2004). However, the fact that the effect of testosterone on carotenoid levels is site-dependent suggests that other factors might be influencing testosterone effects on carotenoids in natural populations. In population 2, grouse abundance was reaching the peak in density while the density in population 1 was lower when the experiment was conducted. Intra-sexual competition (between males) was likely greater in population 2 than population 1. Levels of intra-sexual competition might explain differences in the effect of testosterone on ornamentation, but our sample size ($n = 2$ populations) does not allow us to confirm this idea.

Our results indicate that testosterone increased ornament colouration and size in a similar way in both study sites. However, testosterone increased circulating levels of carotenoids in population 1 but not in population 2. We found that birds challenged with the same number of larvae also ended up with more worms in population 2, suggesting that susceptibility or exposure might be higher in that population. Physiological condition might affect this site-dependent effect. The lack of effect of testosterone on carotenoid levels in population 2 can be explained by a different carotenoid allocation priority, other than ornamentation (i.e. no increase in carotenoids detected). The positive effects of testosterone on carotenoids, and comb colour in population 1 agrees with the idea that those carotenoids, under different circumstances, could be diverted to enhance ornamentation. Thus, we suggest that testosterone may have different effects on individual ornamentation depending on the physiological state of each individual and that carotenoids can be reallocated accordingly. These different allocation priorities can both occur, but the observation that the effect of testosterone on carotenoid levels is site-dependent, lead us to suggest that our study birds were under different environmental conditions at each site, with different physiological states and constraints. Individual-level results support the idea that physiological constraints may alter how carotenoids are allocated. Increases in testosterone were associated with greater increases in comb colouration in population 1, but the opposite occurred in population 2. This suggests that only individuals in population 1 can translate an increase in testosterone levels into a more coloured signal. In

population 2, however, the opposite was found, suggesting that birds might be more physiologically constrained to mirror this testosterone increase towards a more coloured ornament.

Signalling theory, through the handicap mechanism (Zahavi, 1975), posits that the stability of traits conveying information on individual quality relies on the relative costs associated with signal production, which prevent cheating in poor-quality individuals. Our results suggest that the effect of testosterone on ornaments and therefore the relative costs associated with maintenance or production vary depending on the environmental circumstances. The ICHH suggests that only high-quality individuals can afford the immunosuppressive effect of testosterone (Folstad & Karter, 1992). This idea is still under debate and has received mixed support (Roberts *et al.*, 2004). However, most studies have been carried out in captivity (McGraw & Ardia, 2003; Blas *et al.*, 2006) with individuals out of their natural context. We suggest that physiological constraints caused by higher exposure or susceptibility to parasites may alter carotenoid allocation priorities. However, exposure and susceptibility to parasites are two different factors, one related to the abundance of parasites and the other to the individual probability to being parasitized, respectively. Teasing apart these two factors is essential for a deeper understanding of the ICHH and may explain the apparent disparity of results from studies investigating the ICHH (Roberts *et al.*, 2004).

Overall, our results suggest that testosterone and parasites do not have an interactive effect on two components of grouse ornamentation. They suggest, however, that ornament expression and carotenoid allocation priorities are more testosterone-driven than parasite-driven. Delayed effects described for red grouse suggest that the interactive effects might be expressed a year later. In addition, we found that both the effect of testosterone and parasites have a site-dependent effect on redness and comb size, respectively. Considering the site-dependent effect of parasite challenges on parasite infection levels at the end of the experiment, we infer that individuals were in different physiological conditions, which in turn, may have influenced their carotenoid allocation priorities (i.e. increase comb redness or allocate available carotenoids for immune response or overall self-maintenance). Despite the large numbers of studies trying to support the ICHH, there is still considerable debate about whether testosterone-mediated costs define the evolution of such traits (Roberts *et al.*, 2004). Our study suggests that adequately testing the ICHH is a difficult task, given the dynamics of the expression of sexual traits and the context that individuals experience, which should be taken into account. Our work should stimulate more studies that focus on how environmental conditions influence the trade-off between producing or maintaining costly sexual ornamentation and self-maintenance.

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