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Intra-sexual competition alters the relationship between testosterone and ornament expression in a wild territorial bird

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Running head: Ornamentation and social context
Abstract

In a reliable signalling system, individual quality is expected to mediate the costs associated with ornamental displays, with relatively lower costs being paid by individuals of higher quality. These relative costs should depend not only on individual quality, but also on levels of intra-sexual competition. We explored the current and delayed effects that testosterone implants have on bird ornamentation in populations with contrasted population densities, as a proxy for intra-sexual competition. In a replicated experiment, we manipulated testosterone in 196 yearling male red grouse *Lagopus lagopus scoticus* in autumn in populations of high and low levels of intra-sexual competition. Males were assigned to one of three exogenous testosterone (T) treatments: empty implants (T0), small T implants (T1) or larger T implants (T2). We monitored subsequent changes in testosterone levels, ornament size and carotenoid-based colouration, carotenoid levels and body condition from autumn to spring. Testosterone implants increased testosterone levels, comb redness and comb size, and decreased body condition but these effects depended on levels of intra-sexual competition. Specifically, T2-implanted birds increased testosterone levels and comb size more, and reduced condition more, in populations where intra-sexual competition was low. In the following spring, testosterone levels of T2-treated birds kept increasing in populations where intra-sexual competition was high but not in populations where intra-sexual competition was low. Our results highlight that levels of intra-sexual competition alters the relationship between testosterone levels and ornament expression, influencing their condition-dependence; they also indicate that the outcome of standard hormone manipulation conducted in free-living animals vary depending on the population context.

Key words: sexual selection, red grouse, males, hormones, condition-dependence.
Introduction

In many species, males display conspicuous traits considered to be a consequence of inter- and intra-sexual selection processes (Andersson, 1994). Different mechanisms have been proposed to explain the evolution and maintenance of these sexual traits (Maynard Smith and Harper, 2003). Among them, the Handicap Principle suggests that only high quality individuals should be able to afford the costs associated with signal production (Grafen, 1990; Zahavi, 1975). However, males have to compete for resources and the strength of that competition may influence the relative costs associated with the production of sexual traits and therefore the honesty of the signal. Intra-sexual competition is a major selective force in natural populations that strengthen selection on the expression of phenotypic traits (Calsbeek and Cox, 2010). Wild animals have to adjust their resource allocation according to their surrounding environment (Maynard Smith, 1982; Maynard Smith and Harper, 2003). Thus, it is expected that intra-sexual competition may mediate the expression of secondary sexual traits and their associated costs.

Higher levels of intra-sexual competition have been shown to reduce the expression of secondary sexual traits (Vanpé et al., 2007; Vergara and Martinez-Padilla, 2012), possibly to reduce the behavioural and physiological costs associated with the expression of the trait and allowing individuals to successfully survive and reproduce in more competitive environments (Vergara and Martinez-Padilla, 2012). This individual plasticity in the expression of sexual traits may facilitate individuals adjusting the production and maintenance costs of sexual traits according to their surrounding environment, as expected with honest sexual signals. The next issue, however, is to understand how those costs are traded-off when levels of intra-sexual competition differ.

Many animal social signals are testosterone-dependent, and for these traits, testosterone may help to disentangle how social context mediates the signalling costs. The expression of testosterone-dependent sexual traits imposes a cost on individuals, where only high quality individuals are able to stand the costs associated with the expression of the most elaborate displays according to the Immunocompetance handicap hypothesis (Folstad and Karter, 1992; but see
Roberts et al., 2004). Unsurprisingly, testosterone levels in populations increase as intra-sexual competition does, but counter intuitively, testosterone levels of individuals living in more competitive environments are not associated with the expression of secondary sexual traits (Vergara and Martinez-Padilla, 2012) and are poorly linked to individual behaviour (Adkins-Regan, 2005; Fusani, 2008a, b). Thus, it is expected that the costs associated with increased testosterone levels for individuals living in more competitive environments should be higher (Folstad and Karter, 1992; Pérez-Rodríguez et al., 2006). Specifically, when competition for resources increases, individuals might be more constrained and less able to afford testosterone-related costs (Martínez-Padilla et al., 2010). As such, condition-dependence of sexual traits is expected to be stronger in areas of higher competition (Vergara et al., 2012a; Vergara et al., 2012b; Vergara et al., 2012c). If ornaments are costly to produce or maintain (Zahavi, 1975), the cost of producing a testosterone-dependent signal in less competitive environments is expected to be attenuated. Thus individuals can divert resources to other energetically demanding functions. Surprisingly, however, no study has specifically explored how levels of intra-sexual competition influence the costs of producing testosterone-dependent traits.

Studies of sexual selection have mainly focused on ornament size or colour, but a deeper comprehension of the costs associated with ornamental displays requires a focus on different characteristics of the same trait. In birds, most yellow-red sexual traits are pigmented by carotenoids and can be testosterone-dependent (Alonso-Alvarez et al., 2009; Hill and McGraw, 2006; Kurtz et al., 2007; Martínez-Padilla et al., 2010; Mougeot et al., 2007). Carotenoids have immune-stimulant and antioxidant properties and can be deposited in ornaments or used to enhance immune responsiveness (Lozano, 1994; von Schantz et al., 1999), playing a key role in the trade-off between self-maintenance and ornamentation. Testosterone can up-regulate levels of circulating carotenoids, which could buffer testosterone-mediated immunosuppression (Blas et al., 2006; McGraw and Ardia, 2007). However, the testosterone-dependent size and carotenoid colouration of the same ornament do not always provide information on the same individual qualities, possibly due to different
allocation priorities (Martínez-Padilla et al., 2010). Moreover, individuals could change their carotenoid allocation priorities depending on conspecific density (Gautier et al., 2008; Martínez-Padilla et al., 2010). Therefore, if individuals can adjust their testosterone levels to the current social context, and if carotenoid allocation is testosterone-dependent, it is expected that the social context mediates the effect of testosterone on carotenoid allocation priorities (Martínez-Padilla et al., 2010).

In this paper, we explore how intra-sexual competition levels influence current and delayed effects that testosterone has on ornamental displays. We use red grouse (*Lagopus lagopus scoticus*) as a study species, and their supra-orbital combs (size and carotenoid-based colouration) as the focus sexual trait. Male red grouse display red supra-orbital red combs whose size and redness are testosterone and carotenoid-dependent, respectively (Martínez-Padilla et al., 2010; Mougeot et al., 2010; Mougeot et al., 2007). These combs are displayed during intra- and inter-sexual interactions and influence both dominance and mate choice (Redpath et al., 2006a). Red grouse populations cycle, with population density varying from 4- to more than 10-fold during the course of a population cycle and intra-sexual competition has been suggested as a leading hypothesis at explaining red grouse population cycles (Mougeot et al., 2003a). Changes in population density are associated with changes in the social structure of male populations (Mougeot et al., 2005c; Piertney et al., 2008) and changes in the levels of intra-sexual competition between males in autumn (Mougeot et al., 2003a; Mougeot et al., 2003b; Watson, 1985). In fact, it has been shown that intra-sexual competition in male red grouse increases with density with one year as a time lag (Mougeot et al., 2003a; Mougeot et al., 2003b; Piertney et al., 2008). Specifically, as population density increases, competition between males for holding a territory in autumn increases, and successful territory owners have greater survival rates overwinter and breeding outcomes the following spring (Mougeot et al., 2003c; Redpath et al., 2006a; Watson and Moss, 2008). The association between male grouse density and aggressiveness has been consistently reported throughout the range of the species and has been suggested as a proximal factor causing population cycles in red grouse (Martínez-Padilla et al., 2014b).
We manipulated levels of testosterone in young male grouse in autumn in 5 populations, which were all increasing and characterized by different densities, and therefore different levels of intra-sexual competition. Wild males were assigned to one of three exogenous testosterone (T) treatments: empty implants; small testosterone implants or larger testosterone implants. We then explored the current (during autumn, i.e. when implants were delivering the exogenous testosterone) and delayed effects (the following spring, i.e. after the implants were exhausted) of T-treatments on circulating testosterone levels, comb size, comb colour, circulating carotenoids and body condition. Adult males are dominant and more aggressive than yearlings (Mougeot et al., 2005b). In addition, adult and yearling red grouse differ in their survival prospects, breeding success, mating success, parasite levels, body mass and comb size (Mougeot et al., 2006; Redpath et al., 2006a; Seivwright et al., 2005; Vergara et al., 2012a; Vergara et al., 2012b). Thus, we studied only wild yearlings at their time of independence to avoid age-mediated biases (Mougeot et al., 2005a). We initially purged all males of their main parasite to avoid potential confounding effects, because parasites may influence aggressiveness and ornament expression (Martínez-Padilla et al., 2007; Martínez-Padilla et al., 2010; Mougeot et al., 2005b; Mougeot et al., 2007). We predicted that 1) levels of intra-sexual selection should influence the associations between ornament expression and testosterone levels, with birds in populations of high levels of intra-sexual competition being more constrained, and less able to translate increases in testosterone levels into enhanced ornament expression; 2) levels of intra-sexual competition should alter the effect of testosterone on carotenoid allocation to colored ornamental traits. Specifically, we expected a weaker link between ornament colouration and circulating levels of carotenoids under more competitive populations, because carotenoids would be more likely to be used for alternative demanding functions. Finally, we predicted that 3) the expected negative effect of testosterone on individual condition would be less pronounced in populations where intra-sexual competition is low, as males would have more resources to counteract this effect.
Material and Methods

General procedures

The experiment was conducted in autumn 2007 in 5 grouse populations (or moors) in the UK: Edinglassie and Invermark (north-east Scotland) and Catterrick, Moorhouse and Geltsdale (North Yorkshire, England). Further details of the experimental manipulation of testosterone are given elsewhere (Martínez-Padilla et al., 2014a). In autumn, red grouse males start establishing their territories, keeping them overwinter in order to survive and breed in the next spring (Watson and Moss, 2008). The highest mortality rate occurs at the time young male red grouse become independent, in autumn, accounting for around 60% of mortality (Martínez-Padilla et al., 2014a). Holding a territory increases the probability of surviving overwinter for young males (Watson and Moss, 2008). In September 2007 (first capture or R1, Fig. 1 – see appendix), we caught young wild male red grouse (all born in summer 2007) by lamping and netting them at night. 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 a total of 196 yearling males. Upon first capture (R1, Fig. 1) we randomly assigned males to one of three testosterone (T) treatment levels (Htreat): (1) No exogenous testosterone provided (Empty implants for T0-males); (2) intermediate levels of exogenous T (small, 1cm long, testosterone implants for T1-males); (3) higher levels of exogenous testosterone (larger, 2cm long, testosterone implants for T2-males).

Males were kept overnight in individual boxes to collect faecal samples for nematode parasite counts. Prior to release into the wild (early morning), all males were given a 1ml dose of the anthelminthic Levamisole hydrochloride (Nilverm GoldTM, Schering-Plough Animal Health, Welwyn Garden City, UK), a drug effective at purging grouse from their adult Trichostrongylus tenuis nematodes (Hudson, 1986; Mougeot et al., 2005b; Mougeot and Redpath, 2004).

All birds were recaptured one month later (R2, Table 1) and in the following spring (R3, Table 1). In spring, males that survived the previous winter start courtship and mating (Watson and
Moss, 2008). Upon each re-capture event (R1, R2 and R3, Table 2), we estimated worm burdens (see below) and measured comb size, comb redness and took a blood sample to quantify carotenoid and testosterone levels (Table 1). 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 (Mougeot et al., 2005a). Comb redness was measured using a portable spectrophotometer (Minolta CM-2600d, see below). Blood samples (1 mL) from males were collected by intravenous extraction from the brachial vein. Plasma was separated by centrifuging blood for 10 min at 8,000 rpm and stored at −80°C until analyzed for measuring testosterone and carotenoid concentrations.

Parasite abundance.

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. We followed the methods for storing and counting T. tenuis larvae described in previous works (Shaw and Moss, 1989a, b; Shaw et al., 1989). To estimate T. tenuis abundance for each male, we used caecal feaces for T.tenuis egg counts which provides a reliable estimates of worm burdens (see Seivwright et al., 2004).

Testosterone implants

All birds were implanted with two silastic tubes (1.57 mm inner and 2.41 mm outer diameter, Sigma Aldrich Co Ltd, Poole, Dorset, UK) sealed with glue at both ends. Inert-implanted control males (T0 males) were given two empty implants (each one 1cm long), T1-treated males were given two 1cm long implants filled with testosterone and T2-treated males were given two 2cm long implants also filled with testosterone (Sigma Aldrich Co Ltd, Poole, Dorset, UK). Implants were inserted between skin and breast muscles on the flank, under local anesthesia. 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) and they do not raise testosterone levels in birds to pharmacological levels (Mougeot et al., 2005a; Vergara and Martinez-Padilla, 2012).

**Testosterone assays**

We measured plasma testosterone concentration using a commercially available testosterone enzyme immunoassay (Elisa Kit EIA-1559 from DRG Diagnostics, Marburg, Germany), which has been developed and validated for determining testosterone levels in small volumes of avian blood plasma (25 µl) (Washburn et al., 2007). 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 \( (F_{23,59} = 15.99, p < 0.001; r=0.88; \) (Lessells and Boag, 1987). All measurements are given in ng ml⁻¹.

**Carotenoid assays**

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 (10,000rpm for 10 minutes). The supernatant was examined in a ShimadzuUV-1603 spectrophotometer optical density measurements were taken at 446 nm. Plasma carotenoid concentration (µg ml⁻¹) was calculated using a standard curve of lutein (Sigma Chemicals). The standard curve was validated by assessing its parallelism with a serial dilution of plasma samples of the studied species. Note that, although a lutein standard was used to quantify carotenoids in the sample, both lutein and other minor carotenoids present in plasma samples (e.g. zeaxanthin) with overlapping absorbance spectra do also contribute to measured absorbance values. For this reason, we refer to our data as “total carotenoids”. Duplicate sample analyses showed that plasma carotenoids assays were highly repeatable \( (F_{19,20}=602.2, P<0.001, r=0.99)\).

**Comb redness**

We measured comb hue, chroma and brightness using a portable spectrophotometer (Minolta CM-2600D; Konica Minolta, Osaka, Japan). Two colour measurements were taken from the same comb of each male upon each capture. Duplicate measurements were highly repeatable (all > 0.80 and \( p < \)
0.001, Lessells and Boag 1987) and we used average values for the analyses. 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 which wavelengths contribute most to the total reflectance, while chroma is an index of saturation or spectral purity of that colour. These three colour variables were reduced using a principal components analysis (PCA), extracting orthogonal components to be interpreted as indexes of comb redness (PCA - see Martinez-Padilla et al., 2010). The first principal component (PC1) explained 84.1% of comb colour variation (eigenvalue = 1.86), and was positively correlated with hue (eigenvector = 0.54), chroma (eigenvector = 0.57) and brightness (eigenvector = 0.61). We used this PC1 as an index of comb redness, with higher values describing combs that were redder, brighter and with a higher spectral purity.

Levels of intra-sexual competition

Before experiments, we estimated grouse density using a standard playback method previously validated for red grouse (Evans et al., 2007). Briefly, we conducted playbacks at nine points randomly distributed, 250m apart on of each other, and recorded the number of males responding or showing in response. At each point, we played male territorial calls (flight and ground calls) four times, once in each of the four cardinal directions, at full volume using a portable tape player. Following the playback, we scanned the surrounding area with binoculars for five minutes and recorded males that responded by calling and showing within a 100m radius of the survey point. Although the risk of double counting of males is low, we excluded from the analyses those males that were suspected to have been recorded before. Playbacks were conducted in the morning (8:00-12:00) or afternoon (15:00-18:30) avoiding days with strong wind or rain (Evans et al., 2007).

Number of male responses per playback point responding and site were transformed into number of grouse per km$^2$ using the equations provided in Evans et al. (2007). Male grouse density has been shown to be a good proxy of male-male competition (Martínez-Padilla et al., 2014b; Mougeot et al., 2003a). To confirm this in our study populations, we also calculated an index of intra-sexual competition (ISC) dividing the number of male grouse calling by the total number of male grouse
seen per point. This index has been proven to be a good estimator of intra-sexual competition (Vergara and Martinez-Padilla, 2012), and as expected, density and ISC were strongly positively correlated in our study populations (see appendix). We regrouped a posteriori the 5 populations into two classes according to their density or ISC (high versus low density or ISC) as done previously (Calsbeek and Cox, 2010). Once grouped, population densities differed between ISC classes (density: $F_{1,3}=3.63$, $p=0.003$; ISC: $F_{1,3}=2.14$, $p=0.017$). Populations with high intra-sexual competition levels (Catterick, Geltsdale and Edinglassie) had an ISC of 0.89$±$0.37 males calling/males seen, and populations with lower levels (Moorhouse and Invermark) had an ISC had 0.41$±$0.20 males calling/males seen (see appendix). Samples sizes within each population for each experimental group did not allow us to explore patterns within each individual population (Table 2). Populations with high density (Catterick, Geltsdale and Edinglassie) were estimated to have on average 51$±$5 males/Km$^2$, while populations with low density were estimated to have 33$±$4 males/Km$^2$ (Moorhouse and Invermark – see appendix). Thus, independently of the use of ISC or grouse density, both indexes led to the same categorization of each population.

**Statistical analyses**

We used SAS 9.2 for all analyses. We ran separate models for testosterone levels, carotenoid concentration, comb size, comb redness and body condition (body mass being the dependent variable, but with wing length included as a covariate in models, as an index of structural size) as dependent variables. All these variables were fitted to General Linear Mixed Models (GLMM) using a normal error distribution and identity link function. We fitted estimated worm counts to generalized linear mixed models (GLIMMIX) using a negative binomial distribution of errors and log link function. In the analyses of treatment effects, the class factor Htreat (Hormone treatment) had three levels (T0, T1 and T2, see above). To account for the effect of treatments over time, capture time (time) was included as factor with the three time steps considered altogether (R1, R2 and R3). The index of intrasexual competition (ISC) was included as a categorical factor (two classes: high versus low ISC). To account for non-independence of individuals within each population or study site, we included
“individual” nested within “site” as a random factor in all models. We tested for treatment effects on individual changes over time in the above mentioned study parameters by testing for a time × treatment interaction. In addition, we tested whether these treatment effects varied with predicted levels of intra-sexual competition (ISC), by testing for time × ISC and time × treatment × ISC interactions on the dependent variables described above. When significant ISC- and time-by-treatment interactions were found, we further explored those differences by considering separately intra-seasonal changes (within the autumn, ie from R1 to R2) and inter-seasonal changes (from autumn, R2, to spring, R3). Sample size varied during the course of the experiment because some males died or could not be recaptured, or because of missing data (lack of faecal or blood sample). In all models, when interactions were not statistically significant, they were removed, starting with interactions and following a backward selection; therefore the final models were those that best explained the variance of the dependent variables.

To assess variability within individuals, we used GLMM models with “site” included as a random factor. 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 (Martínez-Padilla et al., 2010). We specifically tested whether individual changes in comb colouration or comb size were related to 1) testosterone levels and 2) carotenoid levels, and whether these relationships differed between the hormone treatment groups and ISC levels.

Results

Correlative results

Upon initial capture (R1), testosterone levels, comb size, comb colouration and circulating carotenoids levels did not differ between populations with high and low intra-sexual competition levels (ISC – all p>0.573 – see ESM for further details). None of these variables differed between
treatment groups before the experiment (non-significant ISC×treatment interactions, all p>0.079). However, initial *T. tenuis* parasite abundance (at R1, prior to parasite purging), differed between populations with high and low ISC levels (F$_{1,152}=21.82$, p<0.001), with higher *T. tenuis* abundances in populations with high ISC levels. After parasite dosing (R2), parasites abundance decreased from 276.8±45 (n=47) to 13.5±7 (n=33), and from 921.8±191 (n=90) to 28.0±13.8 (n=39) worms per male in populations with low and high ISC levels, respectively. ISC levels did not influence parasite abundance at R2 (GLM, F$_{1,70}=2.44$, p=0.222), but did so at R3 ($\chi^2=9.52$, p=0.002). Males in populations with high ISC levels then had more parasites than those in populations with low ISC levels (see appendix for further details), irrespective of testosterone treatments ($\chi^2=0.79$, p=0.673).

**Effects of exogenous testosterone manipulations**

Testosterone implants influenced changes over time in testosterone levels, depending on testosterone treatments. As expected, testosterone implants successfully increased testosterone levels (results given in Martínez-Padilla et al., 2014a). In addition, ISC levels influenced the effect of hormonal treatments on changes over time in testosterone levels and body condition (Table 3; significant ISC×time×treatment interactions). Hormone treatments affected comb size and colouration (Table 3, Fig. 1), and they did so similarly in populations with high and low ISC levels (Table 3). Carotenoid levels also changed over time, but these changes were influenced neither by hormone treatments nor by ISC levels (Table 3). We further explored these treatment effects by considering intra-seasonal (within autumn, R1 to R2) and inter seasonal variations (autumn to spring, R2-R3) separately.

**Intra-seasonal ISC-dependent effects of testosterone manipulations**

**Testosterone levels.** Between R1 and R2, testosterone treatment increased testosterone levels (interaction Htreat×time, F$_{2,84}=11.16$, p<0.001) but differently in populations with high and low ISC levels (interaction Htreat×time×ISC, F$_{2,84}=2.85$, p=0.043). Considering T0- and T1-implemented birds,
testosterone levels increased more in T1- than in T0-implanted birds (interaction Htreat*time, F\(_{1,53}=10.68, p=0.001\)), but independently of ISC levels (interaction Htreat*time*ISC, F\(_{1,53}=0.22, p=0.641\)). Considering T0- and T2-implanted birds, however, testosterone levels increased more in T2- than in T0-implanted birds, but this effect depended on ISC levels (interaction Htreat*time*ISC, F\(_{1,66}=2.85, p=0.046\), Table 3). The relative increase in testosterone levels of T2-treated birds was greater in populations with low ISC levels (Fig. 1).

**Comb size and colouration.** Testosterone treatment increased both comb size (interaction Htreat*time, F\(_{2,106}=97.36, p<0.001\). Fig. 1) and comb colouration (interaction Htreat*time, F\(_{2,103}=26.72, p<0.001\)). These increases were similar in T1- and T2-implanted birds for both comb size (F\(_{1,63}=0.06, p=0.800\)) and comb redness (F\(_{1,63}=0.01, p=0.932\)). ISC levels did not influence these treatment effects on comb size or coloration (Table 3).

**Body condition.** During autumn, testosterone treatment influences changes in body condition (interaction Htreat*time, F\(_{2,106}=3.20, p=0.044\), Fig. 1), and these effects depended on ISC levels (interaction Htreat*time*ISC, F\(_{2,106}=5.12, p=0.007\), Fig. 1). Considering T0- and T2-implanted birds first, testosterone treatment decreased body condition (interaction Htreat*time, F\(_{1,83}=4.73, p=0.032\)), irrespective of ISC levels (interaction Htreat*time*ISC, F\(_{1,83}=1.04, p=0.310\)). However, considering T0- and T1-implanted birds, testosterone treatment reduced body condition depending on ISC levels (interaction Htreat*time*ISC, F\(_{1,66}=9.67, p=0.002\)). Testosterone reduced body condition in T1-implanted males, but only in populations with low ISC levels (Fig. 1).

**Inter-seasonal ISC-dependent effects of testosterone manipulations**

**Testosterone levels.** Between autumn (R2) and spring (R3), changes in testosterone levels differed between treatments (interaction Htreat*time, F\(_{2,32}=4.13, p=0.025\), Fig. 1). Considering T0- and T1-implanted birds, changes in testosterone levels did not differ between treatments (interaction Htreat*time, F\(_{1,19}=2.69, p=0.117\)), and ISC levels did not influence changes over time in testosterone levels (interaction Htreat*time*ISC, F\(_{1,19}=0.32, p=0.575\)). However, considering T0- and T2-implanted
birds, changes in testosterone levels differed between treatments depending on ISC levels (interaction Htreat*time*ISC, F_{1,23}=5.80, p=0.024. Fig. 1). In populations with low ISC levels, testosterone levels decreased more in T2 than in T0-implanted birds (interaction Htreat*time, F_{1,18}=23.92, p<0.001), with T2-males ending up with lower testosterone levels than T0-males. In contrast, in populations with high ISC levels, changes over time in testosterone levels did not differ between T2- and T0-implanted birds (interaction Htreat*time, F_{1,10}=0.18, p=0.679, Fig. 1). In populations with high ISC levels, T2-implanted males, which had higher testosterone levels at R2 than T0-implanted males, still circulated more testosterone than T0-implanted males at R3, ie ca. 3 months after the implants were exhausted.

Comb size and colouration. Testosterone-treated birds decreased their comb size between R2 and R3 in relation to control birds (interaction Htreat*time, F_{2,52}=44.06, p<0.001), irrespective of ISC levels (interaction Htreat*time*ISC, F_{2,52}=0.79, p=0.457). This decrease was similar for T1- and T2-implanted birds (interaction Htreat*time, F_{1,32}=3.17, p=0.084), and unaffected by ISC levels (interaction Htreat*time*ISC, F_{1,32}=1.33, p=0.257). Comb colouration decreased in testosterone treated birds (interaction Htreat*time, F_{2,36}=17.24, p<0.001) between R2 and R3, independently of ISC levels (interaction Htreat*time*ISC, F_{2,36}=1.30, p=0.284). Again, this reduction in comb redness was similar for T1- and T2-treated birds (interaction Htreat*time, F_{1,24}=0.53, p=0.472) and unaffected by ISC levels (interaction Htreat*time*ISC, F_{1,24}=3.19, p=0.087).

Body condition. From autumn (R2) to spring (R3), testosterone treatment, ISC levels or their interactions did not influence changes in body condition (all p>0.331).

Seasonal changes in parasites and circulated carotenoid levels

Testosterone treatment did not influence variation in circulating levels of carotenoids between R1 and R2 (Table 3), but changes over time in carotenoid levels depended upon ISC levels (significant ISC*time interaction, Table 3). Circulating levels of carotenoids increased from autumn to spring in populations with low ISC levels (F_{1,11}=18.23, p=0.001), but not in populations with high ISC levels.
Testosterone treatment did not influence parasite abundance (H\text{treat}, F_{1,7}=3.04, p=0.124). However, in spring, parasite abundance depended on ISC levels (F_{1,64}=9.37, p=0.003), being higher in populations with high ISC levels (802±68 worms per grouse) than in those with low ISC levels (243±44 worms per grouse).

**Covariation between testosterone, carotenoid levels and comb redness**

Within autumn (R1-R2), changes in carotenoid levels (ΔCarot) were associated with changes in testosterone levels (ΔT), but depending on ISC levels and hormone treatments (interaction H\text{treat}*ISC*ΔT, F_{2,60}=4.80, p=0.011, Fig. 2). This interaction was significant when comparing T0- to T2-implanted birds (F_{1,51}=7.06, p=0.010), but not when not when comparing T0- and T1-implanted birds (F_{1,35}=0.02, p=0.889). In populations with high ISC levels, changes in carotenoids and testosterone levels were unrelated (F_{1,35}=2.12, p=0.154). In populations with low ISC levels, changes in carotenoids and testosterone levels differed between T2-implanted and control birds (F_{1,16}=3.35, p=0.035), and were positively correlated in control males (F_{1,7}=2.78, p=0.039, estimate: 3.359±2.013), but not in T2-males (F_{1,8}=0.01, p=0.995, estimate: -0.003±0.496).

In addition, changes in comb redness (ΔCred) were associated with changes in testosterone levels (ΔT), also depending on ISC levels and hormone treatments (interaction H\text{treat}*ISC*ΔT, F_{2,71}=3.46, p=0.036, Fig. 2). This interaction was significant when comparing T0- to T2-implanted birds (F_{1,55}=3.62, p=0.009), but not when comparing T0- and T1-implanted birds (F_{1,43}=2.75, p=0.105). Specifically, in populations with high ISC levels, changes in comb redness and testosterone levels did not differ between T2-implanted and control birds (F_{1,38}=0.34, p=0.564). In populations of low ISC, changes in redness and testosterone levels differed between groups (F_{1,17}=3.50, p=0.0389), and were positively associated in control males (F_{1,8}=6.15, p=0.038, estimate: 0.492±0.198), but not in T2-males (F_{1,8}=2.40, p=0.160, estimate: -0.612±0.396). There were no significant interactions between the other variables considered (all p>0.157), neither within autumn, nor between autumn and spring (R2-R3, all p>0.231).
Discussion

Our results highlight that social context, here evaluated in terms of estimated intra-sexual competition (ISC) levels, can influence how ornament expression responds to changes in sexual hormone levels (testosterone). The experiment also suggests that ISC levels can modulate the trade-offs that underlines the expression of ornaments, and the relative costs of producing or maintaining exaggerated ornaments. These findings have further implications for our understanding of the honesty of social and sexual signals. First, we found that intra-sexual competition may mediate the effect of testosterone on body condition in individuals at the time of their independence. Second, the investment in the production or maintenance of sexual signals had delayed costs and effects. And third, population levels of intra-sexual competition can determine the testosterone-dependency of sexual signal components (size, colour), and therefore their value as reliable indicators of individual quality. Overall, our results show that sexual signalling can be better understood when considering a complex set of interactions with time-delayed effects mediated by social context.

Levels of intra-sexual competition have been shown to be tightly associated with grouse density and documented to be the main mechanism that explain population of cycles in red grouse (Moss et al., 1996; Mougeot et al., 2003a) along with nematode parasite abundance (Hudson et al., 1998; but see Redpath et al., 2006b). This might be contradictory given that competition among males may change for a given population density due to the time-lag of aggressiveness in relation to grouse density (Martínez-Padilla et al., 2014b). In this study, all populations were in the increasing phase, explaining why grouse density is a good proxy of intra-sexual competition.

Testosterone, ornamentation and population density.

Testosterone implants successfully increased testosterone levels, within the natural levels of variation known for the model species (Mougeot et al., 2009). However, this effect depended on
intra-sexual competition levels: T2-implanted birds increased their testosterone levels more when intra-sexual competition was lower. The reason why is unclear, although physiological and behavioural reasons may explain this pattern. Individuals in populations of higher intra-sexual encounters might be more physiologically constrained, being unable to raise their testosterone up to the levels individuals in low density populations do. One possibility is that young individuals in such contexts might either down-regulate their levels of testosterone to avoid getting over a physiologically risky threshold, triggering negative feedback mechanisms that decrease the production of endogenous testosterone. It also is possible that males have perhaps metabolized or excreted it at different rates. Unfortunately, our experiment does not allow us to tease apart the mechanistic effect of testosterone implants on suppression of endogenous testosterone versus exogenous testosterone release from R1 to R2.

An alternative, and not mutually exclusive, explanation is that behaviour explains these patterns. Young birds have to find a territory to survive over winter (Watson et al., 1984), so they have to choose the best strategy to acquire this vital resource. Young males in populations of more attenuated intra-sexual competition might increase their testosterone levels to obtain a territory, and because the chances of losing it afterwards are lower due to reduced intra-sexual competition, they might be able to get back to lower testosterone titres the next spring. Also, given that testosterone has been suggested to be linked to aggressiveness and dominance (Mougeot et al., 2005a; Mougeot et al., 2003a), young individuals might be subordinates, with dominant males preventing them from increasing their testosterone levels when competition is higher. In a more competitive environment, young birds might only be able to increase their testosterone levels up to an intermediate level, because they would have to keep fighting the following spring, a cost perhaps not affordable. The observation that testosterone levels in populations with higher levels of intra-sexual competition kept increasing in the following spring, despite the fact that the implants were no longer active, supports this idea. Therefore, we suggest that grouse may show certain plasticity in their territory acquisition strategies according to the competitive environment they are in.
Experimental increases in testosterone levels were mirrored by increases in comb size and comb redness a month after implant, when these were still active and delivering exogenous testosterone. We predicted a weaker link between ornament expression and testosterone levels in birds living in populations of higher levels of intra-sexual competition. Our results agreed with our prediction, although only partially because this effect was also dependent on the amount of exogenous testosterone provided. T2-treated birds increased their testosterone levels more in populations of low levels of intra-sexual competition, but there was no differential effect on comb size or colouration in T2-implanted males when compared to T0-(control) males. This suggests that a similar administration of exogenous testosterone can have a different effect on two components of an ornament (here size and colour) depending on the levels of intra-sexual competition. This agrees with the “dose-response” fallacy suggested when linking hormone levels and behavioural traits (Adkins-Regan, 2005). When looking at inter-seasonal associations between testosterone levels and comb size, we found that these two factors were closely inter-related in populations with low intra-sexual competition only. When competition is higher between males, males kept increasing testosterone levels the following spring, despite the testosterone implants being exhausted within 3 months and therefore no longer active by the following spring. However, their comb size got back to a size similar to that of control (T0-males). Therefore, the relationship between hormone (testosterone) levels and ornament expression is not linear. Rather, there seems to be a threshold level over which testosterone no longer explains comb size variation. It can be speculated that keeping high levels of circulating testosterone in more competitive populations in spring might not be part of a behavioural effect. Alternatively, it is possible that individuals can be more aggressive in spring than inferred from their comb size, as previously suspected (Mougeot et al., 2003b; Seivwright et al., 2005; Vergara and Martinez-Padilla, 2012). We find this explanation plausible because males in more competitive populations have to fight much more to keep their territory and to get a mate in spring. From an endocrine point of view, it has been described that testosterone can be converted into oestrogen by the enzyme aromatase (Hutchison, 1971), particularly abundant in
male songbird brains (Fusani, 2008b; Schlinger and Arnold, 1991). Thus, it might be possible that testosterone levels over a given threshold could trigger other behaviours (e.g., calling behaviours) rather than being associated with ornament maintenance/production. In any case, it is crucial now to establish how the relationship between testosterone levels and comb size, and perhaps also comb colouration, varies not with intra-sexual competition, but with current levels of aggressiveness, in order to better understand the actual mechanism acting in the context of cyclic population dynamics in red grouse as previously assumed (Piertney et al., 2008).

We predicted that intra-sexual competition would influence the bioavailability of carotenoid pigments, depending on the hormone treatments. We found that testosterone treatments did not influence changes over time in circulating levels of carotenoids, but that these depended on intra-sexual competition levels: in populations with high levels of intra-sexual competition, carotenoids levels did not increase over time, from autumn to the following spring, whereas parasite abundance did. In populations with high intra-sexual competition, it is expected that higher intra-sexual encounter rates and behavioural costs may make individuals more susceptible to parasites. Since T.tenuis nematodes reduce carotenoid availability in red grouse (Martínez-Padilla et al., 2007; Mougeot et al., 2007) and food availability or quality have been dismissed as causes to explain population variation in red grouse (Martínez-Padilla et al., 2014b), it is possible that the increase in parasite abundance prevented an increase in circulating carotenoids levels in more competitive populations. At the individual level, our results supported our prediction, as we found that changes in testosterone levels were positively associated with changes in comb redness and carotenoid levels in populations of lower levels of intra-sexual competition in autumn. Proportionally greater increases in testosterone levels were associated with greater increases in carotenoid levels and comb redness in control birds, only when intra-sexual competition was lower. Because of this positive association in control birds, we can hypothesise that, in testosterone-treated birds, carotenoids were used for functions other than signalling, such as immune function (Blas et al., 2006; McGraw and Ardia, 2007) or buffering oxidative stress (Lozano, 1994; von Schantz et al.,
1999), which might be expected since testosterone enhances aerobic metabolism (Buchanan et al., 2001). This suggests another way of explaining how increasing testosterone levels above a certain threshold may not have the expected additive effects on ornament colouration and physiology. The plasticity of the effect of testosterone on carotenoid levels and colouration in birds living in a less stressful environment suggests that males in more competitive populations were more physiologically constrained, making them unable to respond optimally (Martínez-Padilla et al., 2010).

**Condition-dependence of ornamentation**

The “handicap principle” predicts that, in a reliable signalling system, low-quality individuals can pay a relatively greater cost for the same level of signalling than high-quality individuals. Thus, only prime quality individuals, showing the most extravagant secondary sexual traits, should afford the costs of their expression. We predicted that the cost of producing a signal should be higher in more competitive populations. Our results do not seem to support this prediction. We found that T1-treated birds increased their testosterone levels similarly regardless of the levels of intra-sexual competition, but lost body condition only in populations with low intra-sexual competition (Fig. 1). In populations with high intra-sexual competition, all birds worsened their body condition, independently of testosterone treatment. This suggests that the cost of producing intermediate levels of testosterone might be affordable only when competition is low. In T2-treated birds, the increase of testosterone levels was mediated by levels of intra-sexual competition, but changes in body condition were not. This suggests that maintaining high levels of testosterone was an unaffordable cost, independently of the social context. Thus, our results highlights that the costs associated with the production of testosterone-mediated ornaments can be mediated by the amount of hormone produced and the population context.

There are several scenarios under which these results can be framed in. First, we cannot rule out that the cost of ornament expression is paid in other terms that we did not measure. Parasite infection can be such a cost. However, testosterone did not increase *T. tenuis* parasite abundance over the course of the experiment (within a month time, R0 and R1, see Martínez-Padilla et al. 2010)
or until the next spring (at R3; this study), as found in previous experiments (in four months, R1 and R2, see Seivwright et al. 2005). Another possible cost of elevated testosterone, not evaluated in this study, is increased oxidative stress and damage (Mougeot et al. 2009). Second, it has been suggested that sexual selection may operate even in the absence of a production or maintenance cost for the signal (Maynard Smith and Harper, 2003). However, the observation that all individuals lost condition in populations of low levels of intra-sexual competition, does not agree with this idea. Finally, it has been suggested that the exaggeration of sexual display does not have to be necessary linked to heightened condition (Johnstone et al., 2009). Under this scenario, the relationship between the signal and condition may disappear over a given threshold or even become negative (Johnstone et al., 2009). It is likely that individuals living in more or less competitive environments have different resolutions for similar testosterone-mediated trade-offs, leading to different life-history strategies. Further studies looking at resolution of particular life-history trade-offs in different contexts will contribute to a better understanding of honest signalling in wild animals.

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Table 1. Chronogram of the experiment (see material and methods for further details).

| Events                | 1<sup>st</sup> capture (R1) | 1<sup>st</sup> recapture (R2) | 2<sup>nd</sup> recapture (R3) |
|-----------------------|------------------------------|-------------------------------|-------------------------------|
| Dates                 | 25 sept ± 5 days             | 10 Oct. ± 5 days              | 19Feb. ± 5 days               |
| Procedures            | Parasite removal             |                               |                               |
|                       | Hormone implants             |                               |                               |
|                       | (T0, T1 and T2)              |                               |                               |
| Variables measured    | T. tenuis worm abundance     | T. tenuis worm abundance      | T. tenuis worm abundance      |
|                       | Testosterone levels          | Testosterone levels           | Testosterone levels           |
|                       | Comb size                    | Comb size                     | Comb size                     |
|                       | Comb colour                  | Comb colour                   | Comb colour                   |
|                       | Carotenoid levels            | Carotenoid levels             | Carotenoid levels             |
Table 2. Number of male red grouse *Lagopus lagopus scoticus* captured and recaptured over the course of the experiment. The number of birds captured in each population are given in brackets: populations with low intra-sexual competition (IM: Invermark and MH: Moorhouse, respectively); population with high intra-sexual competition (CT: Catterick, EG: Edinglassie and GE: Geltsdale, respectively).

| Intra-sexual competition | Hormone treatment | R1     | R2     | R3     |
|--------------------------|-------------------|--------|--------|--------|
| Low                      | T0                | 17\(8, 9\) | 14\(5, 9\) | 13\(6, 7\) |
| Low                      | T1                | 15\(7, 8\) | 10\(2, 8\) | 9\(4, 5\)  |
| Low                      | T2                | 20\(10, 10\) | 14\(6, 8\) | 11\(6, 5\) |
| **Total (Low)**          |                   | 52\(25, 27\) | 38\(13, 25\) | 33\(16, 17\) |
| High                     | T0                | 49\(30, 9, 10\) | 39\(21, 9, 9\) | 35\(16, 9, 4\) |
| High                     | T1                | 26\(11, 8, 7\) | 23\(8, 9, 6\) | 17\(7, 3, 7\) |
| High                     | T2                | 32\(10, 10\) | 37\(19, 9, 9\) | 35\(25, 6, 4\) |
| **Total (High)**         |                   | 127\(72, 27, 28\) | 99\(48, 27, 24\) | 87\(58, 14, 15\) |
| **TOTAL**                |                   | 179     | 137     | 120     |
Table 3. General Linear Mixed Models testing for the effect of testosterone treatment (treatment) and density on changes over time in testosterone levels, comb size, comb redness and carotenoid levels. When assessing body condition, body mass was the dependent variable, with wing length included in the model as covariate ($F_{1,165}=52.06$, $p<0.001$).

|                      | Testosterone levels | Comb size | Comb colouration | Carotenoid levels | Body condition |
|----------------------|--------------------|-----------|------------------|-------------------|----------------|
|                      | df  | F    | p    | df  | F    | p    | df  | F    | p    | df  | F    | p    |
| Htreat               | 2,118 | 5.00 | 0.008 | 1,170 | 7.86 | <0.001 | 2,145 | 0.95 | 0.389 | 2,118 | 1.14 | 0.324 |
| Time                 | 2,118 | 15.89 | <0.001 | 2,170 | 187.27 | <0.001 | 2,145 | 30.33 | <0.001 | 2,118 | 16.79 | <0.001 |
| Htreat*time          | 4,118 | 4.44 | 0.002 | 2,170 | 39.22 | <0.001 | 4,145 | 14.20 | <0.001 | 4,118 | 1.58 | 0.184 |
| ISC                  | 1,118 | 0.00 | 0.959 | 1,170 | 3.76 | 0.054 | 1,145 | 1.32 | 0.252 | 1,118 | 43.13 | <0.001 |
| ISC*Htreat           | 2,118 | 1.50 | 0.227 | 2,170 | 0.06 | 0.942 | 2,145 | 0.18 | 0.832 | 2,118 | 0.01 | 0.987 |
| ISC*time             | 2,118 | 2.58 | 0.080 | 2,170 | 2.30 | 0.103 | 2,145 | 0.77 | 0.463 | 2,118 | 2.38 | 0.048 |
| ISC*time*Htreat      | 4,118 | 2.76 | 0.030 | 4,170 | 1.48 | 0.211 | 4,145 | 1.49 | 0.208 | 2,118 | 0.87 | 0.485 |

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Fig. 1. Effects of testosterone on ornamentation depending on intra-sexual competition levels.

Changes over time in (mean ± SE) testosterone levels, comb size, comb redness, and body condition (body mass corrected by body size – see methods for further details) according to testosterone treatments and levels of intra-sexual competition. We included bird identity as a random factor in all models. R1 (first capture), R2 (second capture, one month after R1) and R3 (last capture the following spring) denotes the 3 sampling times considered in this study (See appendix and material and methods section for further details). Lines below bars indicate differences between treatments within the same ISC level that were statistically significant (P<0.05 – grey lines highlight differences between T0- and T1-implanted males; black lines highlight differences between T0- and T2-implanted males). Lines above bars indicate differences within pair of treatments among different ISC levels that were statistically significant (P<0.05 – grey lines highlight differences between T0- and T1-implanted males; black lines highlight differences between T0- and T2-implanted males). Note that differences between R1 and R2 denotes intra-seasonal variation and between R2 and R3 inter-seasonal variation.
Fig. 2. Covariation of intra-seasonal changes over time of testosterone levels, carotenoid levels and comb redness in male red grouse according to testosterone treatments and depending on intra-sexual competition levels. Individual changes over time were calculated between R1 and R2 (ie during the autumn) and are shown separately for populations with high (right) and low (left) intra-sexual competition levels.
Highlights

1) We explore how levels of intra-sexual competition influence on male ornamentation
2) Intra-sexual competition mediates the expression of sexual traits
3) It also mediates the link between hormone levels and ornament expression
4) Investment in the production/maintenance of sexual signals had delayed costs
5) Levels of intra-sexual competition influence the reliability of sexual traits