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ORIGINAL PAPER

Ornamental comb colour predicts T-cell-mediated immunity in male red grouse *Lagopus lagopus scoticus*

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Abstract Sexual ornaments might reliably indicate the ability to cope with parasites and diseases, and a better ability to mount a primary inflammatory response to a novel challenge. Carotenoid-based ornaments are amongst the commonest sexual signals of birds and often influence mate choice. Because carotenoids are immuno-stimulants, signallers may trade-off allocating these to ornamental colouration or using them for immune responses, so carotenoid-based ornaments might be particularly useful as honest indicators of immuno-compentence. Tetraonid birds, such as the red grouse *Lagopus lagopus scoticus*, exhibit supra-orbital yellow–red combs, a conspicuous ornament which functions in intra- and inter-sexual selection. The colour of combs is due to epidermal pigmentation by carotenoids, while their size is testosterone-dependent. In this study, I investigated whether comb characteristics, and in particular, comb colour, indicated immuno-competence in free-living male red grouse. I assessed T-cell-mediated immunity using a standardised challenge with phytohaemagglutinin. Red grouse combs reflect in the red and in the ultraviolet spectrum of light, which is not visible to humans but that grouse most likely see, so I measured comb colour across the whole bird visible spectrum (300–700 nm) using a reflectance spectrometer. I found that males with bigger and redder combs, but with less ultraviolet reflectance, had greater T-cell-mediated immune response. Comb colour predicted T-cell-mediated immune response better than comb size, indicating that the carotenoid-based colouration of this ornament might reliably signal this aspect of male quality.

Keywords Sexual selection · Carotenoid · Cellular immunity · Phytohaemagglutinin · Tetraonid bird

Introduction

Brightly coloured or exaggerated sexual ornaments might function as reliable signals of individual quality and often assume intra- or inter-sexual functions (dissuading other competitors or facilitating mate choice; e.g. Hamilton and Zuk 1982; Andersson 1994). Hamilton and Zuk (1982) suggested that female preferences for males with the most exaggerated sexual traits evolved because ornament expression might signal the genetic ability of males to resist parasite infections. Females could benefit by choosing a mate with fewer parasites or whose ability to cope with parasites will be passed onto offspring (Hamilton and Zuk 1982; Møller 1990; Zuk 1992; Andersson 1994; Møller et al. 1999).

The role of parasites in sexual selection has been particularly well studied amongst birds. However, the relationships between ornamentation and parasites are often inconsistent (see Folstad and Karter 1992; Møller et al. 1999; Getty 2002), and measures of host immune responses have been suggested to provide more accurate indications

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of the role of parasite-mediated sexual selection than estimates of parasite intensity (Møller and Saino 1994; Zuk 1996; Møller et al. 1998, 1999). Indeed, several studies have shown that sexual ornaments signal immuno-competence, i.e. the ability of an individual to produce an immune response to pathogens, indicating that sexual signals might reliably indicate this aspect of male quality (Zuk et al. 1995; Zuk 1996; Saino et al. 1997; Møller et al. 1999; Duffy and Ball 2002). The relationship between ornaments and immuno-competence also appears to be stronger than that between ornaments and parasites (Møller et al. 1999).

Recently, plumage and integument colouration produced by carotenoid pigments has received particular attention (e.g. Hill and McGraw 2006). Carotenoids determine the bright red–yellows of many sexual traits and are among the most familiar targets of female choice (Hill 2002; Hill and McGraw 2006). Animals cannot synthesise carotenoids but must ingest them, so diet may limit ornament expression (Olson and Owens 1998; Hill and McGraw 2006). Moreover, carotenoids are powerful antioxidants and immuno-stimulants (Møller et al. 2000). Individuals can allocate available carotenoids to ornaments or self maintenance, and the resulting trade-offs may confer honesty on sexual signals (von Schantz et al. 1999; McGraw and Ardia 2003; Blas et al. 2006). Because of the immuno-stimulant properties of carotenoids, carotenoid-based signals should be particularly useful as honest indicators of immuno-competence (e.g. Faivre et al. 2003a; McGraw and Ardia 2003; Blas et al. 2006).

In this study, I investigated whether carotenoid-based ornamentation predicted T-cell-mediated immunity (CMI; the ability to mount a primary inflammatory immune response) in a free-living Tetraonid bird. I used the phytohaemagglutinin (PHA) skin test to measure CMI, which consists of challenging the immune system through the subcutaneous injection of an innocuous plant lectin. This test follows a long-established protocol developed in poultry science (Goto et al. 1978) and provides a measure of T-cell-mediated recruitment of circulating leucocytes (Martin et al. 2006; Kennedy and Nager 2006). The immediate response to the PHA injection is a local inflammatory response encompassing increased blood supply and cellular infiltration into the damaged tissue. In birds, the first cell type to arrive in inflammation is usually heterophils, followed by other cell types, including those directly involved in antigen presentation to the acquired immune system (Martin et al. 2006; Kennedy and Nager 2006). PHA-induced swelling is likely to measure aspects of both innate and acquired cellular immunity, with a different time course (Kennedy and Nager 2006). I measured wing web swelling 24 h after PHA exposure, when it is likely that only the first phase of the response is underway and the infiltration by T cells is more likely to be a relatively minor, but increasing, component of the cellular response (see Kennedy and Nager 2006).

Tetraonid birds, such as the red grouse *Lagopus lagopus scoticus*, exhibit brightly coloured supra-orbital combs, a main sexual ornament that functions in both intra-sexual competition and mate choice: males with bigger and redder combs are typically in better condition, dominant and more aggressive and have a greater pairing success (Moss et al. 1979; Mougeot et al. 2004, 2007a; Redpath et al. 2006). The size of male combs is testosterone-dependent (Mougeot et al. 2004, 2005a, b) and their orange–red colouration is due to epidermal carotenoid pigmentation (Egeland et al. 1993; Mougeot et al. 2007a). Previous works showed that male red grouse with bigger combs are more immuno-competent (Mougeot et al. 2004). Comb size and comb colour, however, are not independent, as males with bigger combs usually also have redder combs (Mougeot et al. 2007a). Whether comb colour advertises T-cell immune responsiveness and the relative importance of comb size and colour as predictors of this individual quality remain unknown. Red grouse combs reflect both in the red and in the near-ultraviolet (UV), which is invisible to humans but that grouse most likely see (Siitari and Viitala 2002). I thus measured comb colour using a reflectance spectrometer, across the whole avian visible spectrum. Recent work showed that redder combs have less UV reflectance because the epidermal carotenoid pigmentation acts as a mask to reduce background UV reflectance by the dermis of combs (Mougeot et al. 2007a). I thus predicted that bigger and redder combs, but with less UV reflectance, would indicate a greater cellular immune response (greater swelling 24 h after PHA exposure). I also investigated which comb characteristics (testosterone-dependent comb size vs carotenoid-based comb colour) best predicted immune responses.

Methods

Captures and measurements In autumn 2001 (9–16 October 2001), I caught 23 males on Edinglassie estate, a grouse moor in NE Scotland, by lamping and netting them at night (Hudson 1986). Each was individually ringed and aged from plumage and morphology. Males were classified as young, i.e. born in the previous summer, or old, i.e. >1 year old. I measured the maximum length and height of flattened combs with a ruler (nearest 1 mm) and calculated comb area (comb width × height) as a measure of ornament size (see Mougeot and Redpath 2004; Mougeot et al. 2005a, b). I measured wing length with a ruler (nearest 1 mm) and weighed each male with a Pesola (nearest 5 g). I calculated the condition index of weight corrected for size using the residuals from a generalised linear model (GLM) of body mass on wing length ($F_{1,21}=10.45$; $P=0.004$; see Mougeot et al. 2005a, b). I then measured comb colour using a reflectance spectrometer, assessed CMI (see below) and released males back to the wild.

Comb colour measurements I measured comb colour of males in the field using a S-2000 reflectance photometer (Avantes, Eerbeeck, the Netherlands) connected to a laptop computer, both being powered by a field generator. Grouse combs are perceived by humans as red due to the presence of carotenoids (Mougeot et al. 2007a), but inclusion of the UV (300–400 nm) is necessary because grouse are most likely sensitive to UV light (Siitari and Viitala 2002). Male combs were subjected to a DH-2000-FHS deuterium–halogen light source (Ocean Optics) with a spectral range from 280 to 800 nm. Comb reflectance was measured with a 45° to normal fibre-optic that provides illumination from the light source and transfers reflected light to the spectrometer. Measurements were taken in dark conditions (at night), with the whole probe surface being carefully placed against the comb surface. The data were converted into digital information using a DAQ Card 700, and reflectance data were calculated relative to a Spectralon® 99% white standard reference using the Spectrawin 3.1 software. Reflectance values were obtained at 0.4-nm intervals between 280 and 800 nm. I took two measures for each male. Reflectance data were highly and significantly repeatable (repeatability values>0.93; all $P<0.001$). The spectra obtained for each bird were averaged and mean reflectance was summarised over 10-nm steps for statistical analyses.

Colour variables The reflectance spectrum of grouse combs is double-peaked (Fig. 1), and such spectra are sometimes difficult to describe in terms of hue and chroma (Cuthill et al. 1999). I thus summarised the spectra using two complementary approaches.

First, the following colour variables are calculated for each male in the interval 300–700 nm, which is the range of avian sensitivity (see Endler 1990): (1) total brightness (sum of reflectance in the interval 300–700 nm); (2) UV chroma (reflectance in the interval 300–400 nm, in percent, relative to total brightness); (3) red chroma (reflectance in the interval 600–700 nm, in percent, relative to total brightness); (4) UV hue (wavelength, λUV, at which maximal reflectance was reached in the interval 300–400 nm, Fig. 1); and (5) reflectance midpoint (λvis50), which is the wavelength half way between that of maximal (λmax) and minimum (λmin) reflectance in the interval 400–700 nm; Fig. 1). Total brightness, UV chroma and red chroma measures relate to the amount of incident light that is reflected by combs across the whole spectrum, in the UV and in the red, respectively. λUV and λvis50 are common measures of spectral location for UV and carotenoid-based colours, respectively. A greater λvis50 value indicates redder combs.

Second, I used a principal component analysis (PCA) of original reflectance data of all males. This method makes no assumptions about how reflectance variation is perceived or which aspects of the spectrum might be important (Bennett et al. 1997; Cuthill et al. 1999). Because brightness variation between spectra is often large and not particularly meaningful (Cuthill et al. 1999), I standardised spectra for brightness before conducting the PCA analysis.

Assessment of CMI I used the PHA (SIGMA L-8754) skin test to measure CMI. This test has been routinely applied in avian field studies (Smith et al. 1999) and consists of an intra-dermal injection of PHA, which produces a prominent perivascular accumulation of T-lymphocytes followed by

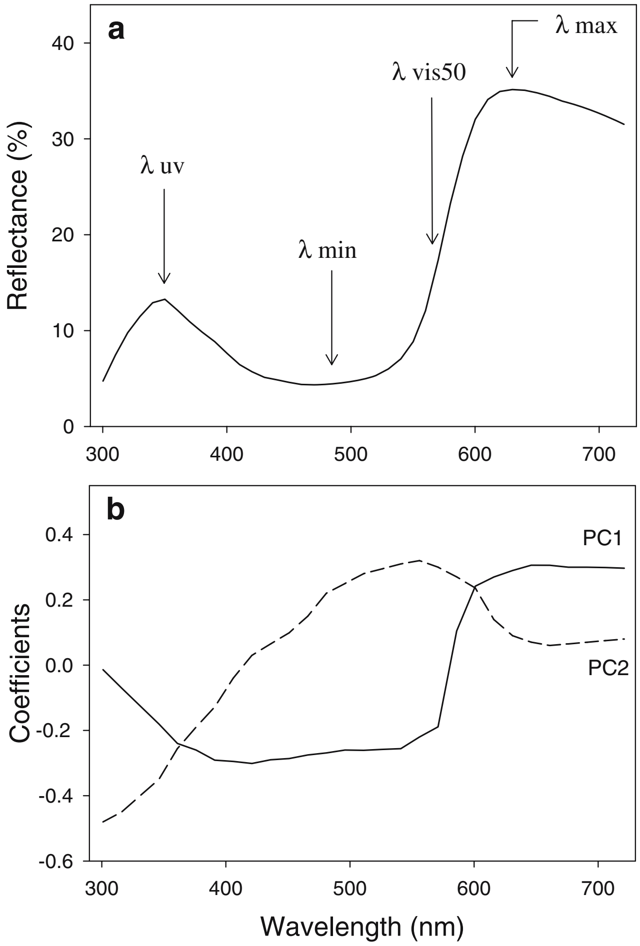

Fig. 1 **a** Reflectance spectra of male red grouse combs (mean reflectance, in percent, $n=23$ males). Spectral location variables used in the analyses included λUV (wavelength at which maximum reflectance was reached in the UV interval 300–400 nm) and λvis50 or reflectance mid-point [wavelength half-way between that at which maximum reflectance (λmax) and minimum reflectance (λmin) were reached in the visible interval (400–700 nm)]. **b** Coefficients relating the first two principal components (PC1–PC2) to the original reflectance spectra ($n=23$ males). See "Methods" for details

Table 1 Loadings for the first two principal components (PC1 and PC2) of a PCA on original comb reflectance data (coefficients in Fig. 1b) and other comb colour variables

	Principal components	
	PC1	PC2
Eigenvalue	10.36	2.92
Variance explained (cumulative)	69.1%	88.5%
Correlation with		
Comb λUV	−0.33	+0.71
Comb λvis50	+0.81	−0.30
Comb UV chroma	−0.61	−0.77
Comb red chroma	+0.981	+0.08
Comb total brightness	+0.020	+0.28

macrophage infiltration (Goto et al. 1978). For the test, each male was injected with 0.1 ml of physiological saline solution [phosphate-buffered saline (PBS)] at a marked site on the right wing web (control) and with 0.2 mg of PHA in 0.1 ml of PBS (challenge) on the left wing. I measured the web thickness at each injection site four times prior to injection and 24 h (±10 min) after injection with a pressure-sensitive dial thickness gauge (to the nearest 0.01 mm). Wing web thickness measures were highly and significantly repeatable (R=0.83; P=0.008). CMI response was calculated as the change at 24 h in average thickness of the left wing web (PHA test) minus that of the right wing web (control). In most birds, the maximum response to PHA is 24 h post challenge and the response disappears after 48 h (Smith et al. 1999). In red grouse, there is little swelling 12 h post injection (unpublished data) but significant swelling 24 h post injection, consistent with a greater response around 24 h after challenge. CMI responses can show diurnal variations (e.g. Martinez-Padilla 2006), but I controlled for this potential source of variation by injecting all males at night at 2300–2400. For this procedure, males were kept in pens provided with food (heather) and water.

Statistical analyses I used SAS 8.01 (SAS 2001). I used the Princomp procedure (SAS 2001) for the PCA of reflectance data. I investigated natural correlates of CMI using GLMs. The dependent variable (Log-transformed wing web swelling, in millimeters) was fitted to models using a normal distribution and identity link function. I used the REG procedure and a backward selection of variables using the AIC criteria to identify which comb characteristics best predicted comb size. All tests are two-tailed.

Results

Reflectance spectra of red grouse combs

The average reflectance spectrum of red grouse combs is shown in Fig. 1a (n=23 males). I summarised reflectance data by conducting a PCA of the original reflectance spectra (Table 1; Fig. 1b). The first principal component (PC1) had negative loadings across the wavelength range 300–600 and positive loadings for longer wavelengths >600 nm (Fig. 1b). PC1 correlated negatively with UV chroma but positively with red chroma and reflectance midpoint (Table 2). The second principal component (PC2) had positive loadings for medium wavelengths (450–600 nm) and negative coefficients for shorter wavelengths <400 nm (Fig. 1b), and negatively correlated with UV chroma but positively correlated with λUV (Table 2).

Comb colour was not independent of comb size. Red chroma (r=0.46; P=0.025) and λvis50 (r=0.59; P=0.003) positively correlated with comb size, while UV chroma, λUV and total brightness were not significantly related to comb size (all P>0.169). PC1 positively correlated with comb size (r=0.51; P=0.012), but PC2 was not significantly related with comb size (r=−0.14; P=0.530). Bigger combs were thus also redder.

Table 2 Summary statistics for the regression analyses (GLM) of T-cell-mediated immune response (log-transformed wing web swelling) on comb characteristics (univariate analyses on each comb variable and on the first two principal components summarising comb reflectance data; see results and coefficients in Fig. 1b)

Regression of X on Y	Parameter estimate (slope ± SE)	F value	df	P value	R^2
CMI*					
Comb size	+0.003±0.001	5.96	1,21	0.035	0.23
Comb λUV	−0.006±0.006	1.31	1,21	0.267	0.06
Comb λvis50	+0.010±0.004	6.56	1,21	0.018	0.24
Comb UV chroma	−0.024±0.009	7.47	1,21	0.013	0.26
Comb red chroma	+0.017±0.005	12.57	1,21	0.002	0.37
Comb brightness	−0.000±0.000	0.51	1,21	0.478	0.02
PC1	+0.0336±0.009	12.22	1,21	0.002	0.37
PC2	+0.016±0.023	0.73	1,21	0.474	0.03

Significant predictors are highlighted in bold

Comb colour, comb size and cell-mediated immunity

Variation in CMI (wing web swelling at 24 h) was not significantly explained by age (GLM: $F_{1,21}$=0.64; P=0.426) or sampling date ($F_{1,21}$=0.30; P=0.792), but was explained by condition: males in better condition had greater CMI ($F_{1,21}$=8.28; P=0.009).

Univariate analyses indicated that a greater CMI was predicted from bigger and redder combs (greater red chroma and greater λvis50) with less UV reflectance (lower UV chroma; Table 2; Fig. 2). Similar results were obtained using the first two principal components summarising the original reflectance data. CMI significantly positively correlated with PC1 but was not significantly related to PC2 (Table 2; see Fig. 1b).

To evaluate which comb characteristics best predicted CMI, I further conducted a multiple regression of CMI on comb traits and a backward selection of explanatory variables. In a first analysis, I included comb size and the comb colour variables λUV, λvis50, UV chroma, red chroma and total brightness. The predictors of CMI retained in the model (P=0.150 level) were red chroma ($F_{1,20}$=12.80; P=0.002; parameter estimate: +0.013±0.005) and comb size ($F_{1,20}$=2.65; P=0.119; parameter estimate: +0.0011±0.0007). In a second analysis, I included comb size and the first two principal components summarising the reflectance data. The predictors kept in the model were PC1 ($F_{1,20}$=12.22; P=0.002; parameter estimate: 0.039±0.009) and comb size ($F_{1,20}$=2.60; P=0.122; parameter estimate: +0.034±0.011). In both analyses, comb colour best predicted CMI while comb size did not significantly (P=0.119 and P=0.122, respectively) explain CMI after comb colour.

Because CMI positively correlated with condition, I also tested whether variation in CMI was predicted from comb characteristics after controlling for condition by including this variable as a fixed effect in all models. Greater CMI (relative to male condition) was significantly predicted from comb size ($F_{1,20}$=8.85; P=0.008), red chroma ($F_{1,20}$=16.16; P<0.001), UV chroma ($F_{1,20}$=6.68; P=0.018) and λvis50 ($F_{1,20}$=6.98; P=0.016), PC1 ($F_{1,20}$=16.87; P<0.001), but not from other comb characteristics (all P>0.200).

Discussion

Comb colour as an indicator of male CMI

In male red grouse, I found that T-cell-mediated immune responsiveness (PHA induced swelling 24 h after injection) could be well predicted from comb characteristics, and in particular from comb redness, which is due to carotenoid pigmentation (Mougeot et al. 2007a). The intensity of the CMI response measured here at 24 h, and predicted from comb colour, was most likely mainly the first phase of the cellular response (inflammation and infiltration of heterophils into the damaged tissue; see Kennedy and Nager 2006). For the experiment, birds were kept for 24 h, and this procedure is most likely stressful. It is possible that some birds, in particular those in poor condition, stressed more than others during the procedure, which could have affected their CMI responses. I measured comb colour and condition prior to challenge and found that both initial condition and comb colour predicted the CMI response 24 h later. I also found that initial comb colour predicted CMI after controlling for initial condition. If stress susceptibility and cellular immune responses are tightly related in red grouse, comb colour might primarily reflect an individual's ability to cope with stress and, secondarily, its ability to raise an immune response to a standardised challenge.

The reflectance of red grouse comb peaks in the UV and in the red part of the spectrum (Fig. 1). Grouse most likely see in the near UV, as demonstrated by behavioural

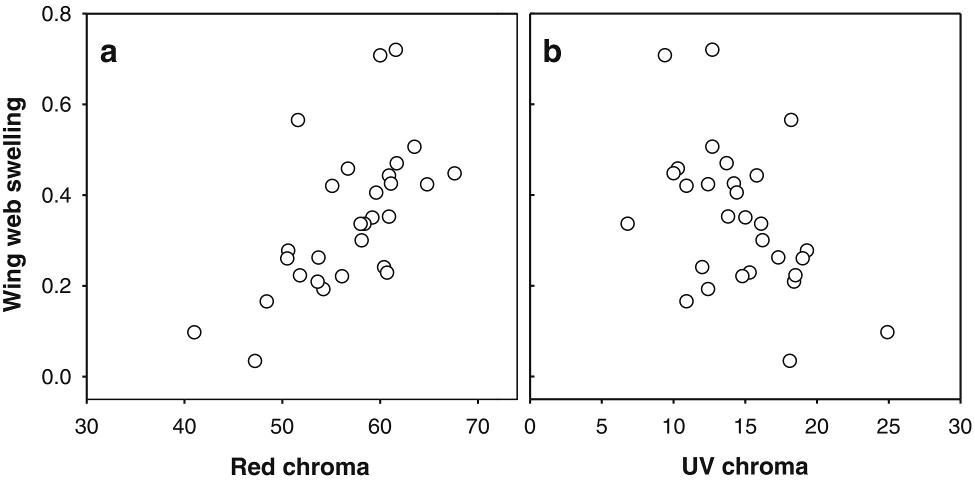

Fig. 2 Relationships between CMI (wing web swelling) and **a** red chroma and **b** UV chroma of male red grouse combs

experiments on another grouse species, the black grouse *Tetrao tetrix* (Siitari and Viitala 2002). In red grouse, a greater CMI was predicted from combs with less, not more, comb UV reflectance and from redder combs (greater red chroma and λvis50). This was confirmed by results using the PCA of reflectance data. The PC1, which opposed comb redness (red chroma and λvis50) and UV reflectance (UV chroma), significantly predicted CMI. Carotenoid pigments are present in lipid droplets in the epidermal surface of combs and are responsible for their red colour (Hollett et al. 1984). The UV, however, is due to reflectance by the dermis of combs, which acts as a background to the red pigmented surface of combs (Mougeot et al. 2007a). Thus, the UV reflective background might expose a lack of carotenoid pigmentation and thereby influence signal perception to a receiver with UV vision.

Honest carotenoid-based signalling

Carotenoid-based ornaments have been found to indicate immuno-competence in other birds, like, for instance, in the zebra finch (McGraw and Ardia 2003; Alonso-Alvarez et al. 2004), mallard (Peters et al. 2004) or European blackbird (Faivre et al. 2003b). Signalling theory predicts that signals of individual quality should be costly to ensure the honesty of the signalling system and prevent poor-quality individuals from cheating (Zahavi and Zahavi 1997). Carotenoid-based signals might be particularly useful as an honest indicator of male immuno-competence for two main reasons. First, because carotenoids cannot be synthetised de novo by vertebrates, but must be acquired through diet, only good foragers would accumulate enough carotenoids to show bright colours (Hill and McGraw 2006). Moreover, immuno-competence is often condition-dependent (Sheldon and Verhulst 1996), and good foragers are also expected to be in better condition and, therefore, to have more resources to allocate to respond to infections and immune challenges. Male red grouse in better condition typically have bigger and redder combs (Mougeot and Redpath 2004; Mougeot et al. 2007b). CMI is also condition-dependent (males in better condition had greater CMI). However, comb characteristics predicted well CMI even when condition was controlled for. Second, carotenoid pigments have important physiological properties, and these might play a key role in ensuring honest signalling of health and immuno-competence. Carotenoids are potent antioxidants and immuno-stimulants (Møller et al. 2000), so more circulated carotenoids would allow for greater immune responsiveness. For instance, in captive birds, carotenoid supplementation via diet has been shown to increase immune function and, in particular, T-cell-mediated immune responses (McGraw and Ardia 2003; Alonso-Alvarez et al. 2004). Immune activation has also been shown to have the opposite effect, i.e. reducing circulating carotenoids and ultimately reducing carotenoid-based ornamentation (e.g. Faivre et al. 2003a; Alonso-Alvarez et al. 2004).

Male red grouse with redder combs (more carotenoid pigmentation) have more circulating carotenoids, as shown in a recent study (Martinez-Padilla et al. 2007) and as found in several other bird species (reviewed in McGraw 2006). The carotenoid-based colouration of red grouse combs might thus directly and reliably signal cell-mediated immunity and male health because of the immuno-stimulatory action of ingested and circulated carotenoid pigments, and because of allocation trade-offs, individuals can either use these pigments to boost their immune system or to pigment their ornaments. A higher quality individual would be able to do both, i.e. show bright colour without compromising immunity.

Bigger combs also predicted a greater CMI response, consistent with a previous study (Mougeot et al. 2004), but two lines of evidence indicated that comb redness predicted CMI better than comb size. First, the strongest relationships were found between CMI and red chroma and PC1 (Table 2). Second, multiple regression analyses retained red chroma or PC1 as significant predictors of CMI, while comb size did not significantly explain CMI after comb colour (red chroma or PC1). In red grouse, males with bigger (and redder) combs have more circulating testosterone (Mougeot et al. 2005a, b) and also have greater cellular immune response, despite elevated testosterone being associated with reduced cellular immunity (Mougeot et al. 2004). This might be because males with bigger (and redder) combs can afford having more testosterone without compromising immuno-competence because they are of higher quality and in better condition (see Mougeot et al. 2004). Here, I found that carotenoid-based comb redness predicted cellular immunity better than testosterone-dependent comb size, although both are inter-related. Recent works have highlighted that testosterone and carotenoid-based signals might not be independent because testosterone can increase carotenoid availability and increased circulating carotenoid levels could buffer the immuno-suppressive effects of testosterone (Blas et al. 2006; Mougeot et al. 2007b). Further experimental work on the possible interactions between testosterone, carotenoids and ornamentation might improve our understanding of the mechanisms ensuring honest sexual signalling.

In conclusion, male red grouse with bigger and redder combs with less UV reflectance had greater cell-mediated immunity and were likely to be of higher phenotypic quality. Females might therefore prefer males on the basis of their comb characteristics, and in particular their carotenoid-based colouration, for their higher immuno-competence, which might underline a better ability to cope with parasite infections.

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