Parasites, condition, immune responsiveness and carotenoid-based ornamentation in male red-legged partridge *Alectoris rufa*

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Brightly coloured or exaggerated ornaments can dissuade competitors and facilitate mate choice, and might reliably advertise individual quality (Darwin 1871, Andersson 1994). Plumage and integument ornamental colouration produced by carotenoid pigments has received particular attention (e.g. Hill and McGraw 2006). Carotenoids determine the bright yellow-reds of many sexual traits, and are among the most familiar targets of female choice (Hill 2002, Hill and McGraw 2006). Vertebrates cannot synthesize carotenoids *de novo*, but must ingest them, so diet may limit ornament expression (Brush 1981, Goodwin 1984). Carotenoid signals might thus be indicators of foraging efficiency and condition: good foragers would be able to acquire more carotenoids and therefore express more intense coloration (Endler 1983, Hill and Montgomery 1994, Hill and McGraw 2006). In addition, carotenoids act as antioxidants and immune-enhancers (Burton 1989, Olson 1989, Olson and Owens 1998, Möller et al. 2000). Because of these physiological functions, individuals face the trade-off between allocating available carotenoids to self-maintenance versus ornamental coloration, which could further confer honesty to carotenoid-based signalling (Lozano 1994, von Schantz et al. 1999, McGraw and Ardia 2003, Blas et al. 2006). If only healthy males can afford the immunological cost of diverting available carotenoids to ornament expression, carotenoid-dependent ornaments could honestly signal the health status of males (Lozano 1994, von Schantz et al. 1999). By basing their mate choice on such ornaments, females would mate with healthier males.

Carotenoid-based ornaments should be particularly sensitive to immune challenges and to parasite infections (Lozano 1994). Intestinal parasites can damage the gut epithelium and thereby reduce carotenoid absorption (Allen 1987) or may compete with hosts for ingested carotenoids (Czeczuga 1980). Parasites also often reduce condition (e.g. Hudson et al. 2001), and might force hosts to mobilise available carotenoids for immune responses to the detriment of ornamental coloration (Lozano 1994). As a result, a negative relationship might be expected between parasite abundance, circulating carotenoids and coloration.

In this study, we investigated whether carotenoid-based ornamentation predicted condition, as indexed by body mass corrected for size, immune responsiveness or parasite load, and the relationships between circulating carotenoids, body condition, immune responsiveness and parasites in young male red-legged partridges *Alectoris rufa*. We used the Phytohaemagglutinin (PHA) skin test to measure
immune responsiveness, which consists of challenging the immune system through subcutaneous injection of a plant lectin (Goto et al. 1978, Smith et al. 1999). A medium-sized, monogamous Galliform, the red-legged partridge exhibits conspicuous orange-red eyes and beak, which are pigmented by carotenoids (Blas et al. 2006). Three lines of evidence indicate that these carotenoid-based ornaments function in inter-sexual selection: (1) males have redder ornaments than females (Villafuerte and Negro 1998); (2) plasma carotenoid levels and carotenoid-based ornamentation are maximal during the mating season (Negro et al. 2001), and (3) wild red-legged partridges mate assortatively based on carotenoid-based ornamentation (Mougeot et al., unpubl. data).

Here we hypothesised that when food is not limited, carotenoid-based ornaments primarily indicates a male’s current health and immune response capacity. We studied captive birds, which were fed ad libitum on a constant diet with known carotenoid content, allowing us to minimize variation due to differences in access to food and carotenoid pigments. Red-legged partridges are often infected by coccidia (Villanúa et al. 2006), which can negatively impact condition of avian hosts. We predicted condition, plasma carotenoid concentration and immune responsiveness would be negatively associated with coccidia abundance. We also predicted carotenoid-based ornamentation would reflect immune responsiveness and abundance of coccidia rather than condition.

Methods
Study birds
We conducted this study on captive red-legged partridges held at Dehesa de Galiana, a facility of the Instituto de Investigación de Recursos Cinegéticos (IREC, Ciudad Real, central Spain). We isolated 42 sexually mature males partridges (born in spring 2006) the 30TH of November 2006 and individually housed them in outdoor cages (see Pérez-Rodríguez et al. 2006). Birds were fed ad lib with commercial food pellets containing 20% protein, 4.5% fat, 3.7% cellulose and 5.26 μg of carotenoids (96% lutein) per g of food. We collected all data 11–12TH of December 2006, after a 1-month period of acclimatation to individual cages.

Measurements
For each male, we measured body mass with a Pesola scale (± 5g) and tarsus length with a digital calliper (± 0.1 mm). We calculated the condition index of (log-transformed) body mass corrected for (log-transformed) tarsus length, as an index of structural size ($F_{1,40} = 7.65$, $P < 0.009$), similar to previous works with this species (Blas et al. 2006, Bortolotti et al. 2006).

Digital photographs
We used digital photographs to measure the red coloration of the beak, nostril and eye ring of males. For each male, we took a digital picture (2,272 × 1,704 pixels) of the left side of the head (see Fig. 1) on the 11th of December 2006. All pictures were taken using the same digital camera (Nikon Coolpix 4500) and under standardized conditions (same fluorescent light illumination and grey background, same distance (40 cm) between the body part to analyse and the camera). For each picture, we placed the same grey standard reference (Kodak Gray Scale, Kodak, New York) near the body parts that were measured. To determine repeatability of colour measurements, we took a second picture for a sample of 20 males.

Carotenoid-based ornamentation
We analysed the digital pictures using Adobe Photoshop v7.0. From each picture, we measured the RGB components (the average intensity of Red, Green and Blue components of pixels) of the eye ring, beak (upper mandible) and nostril, and of the grey reference (see Fig. 1). We considered the eye ring, beak and nostril colours separately because the eye rings and nostrils are soft tissues, whereas the beak is a more keratinized structure. We analysed the digital pictures using Adobe Photoshop v7.0. From each picture, we measured the RGB components (the average intensity of Red, Green and Blue components of pixels) of the eye ring, beak (upper mandible) and nostril, and of the grey reference (see Fig. 1). We considered the eye ring, beak and nostril colours separately because the eye rings and nostrils are soft tissues, whereas the beak is a more keratinized structure. The intensity of carotenoid-based red coloration, or RGB value (hereafter referred to as “redness”) was calculated as $R$ divided by the average of $R$, $G$ and $B$ (Villafuerte and Negro 1998). RGB values of the grey reference were used to standardize all colour measurements and correct for possible differences in coloration between pictures. In addition, we calculated the relative amount (% pixels) of the eye ring area pigmented by carotenoids (hereafter referred to as eye ring pigmentation; see Fig. 1c, d). From the digital pictures, we thus obtained the following colour measurements for each male: (1) beak redness, (2) nostril redness, (3) eye ring redness, and (4) eye ring pigmentation (% of eye ring area pigmented). We assessed measurement repeatability following Lessells and Boag (1987). All colour measurements were repeatable (repeatability values of 0.82, 0.73, 0.80 and 0.72, respectively, all $P < 0.01$, n = 20).

Blood sampling and plasma carotenoid analysis
For each male, we took a blood sample (c. 800 μl) from the brachial vein of the right wing using a heparinized syringe. Blood was stored at 4° C and centrifuged at 10,000 rpm within 4 hours of collection. Plasma was then stored at −80° C until analysis. Carotenoids were quantified by diluting 60 μl of plasma in acetone (1:10 dilution). The mixture was vortexed and centrifuged at 10,000 rpm for 10 min to precipitate proteins. The supernatant was examined in a Shimadzu UV-1603 spectrophotometer and we determined the optical density at 446 nm, the wavelength of maximal absorbance for lutein (Mínguez-Mosquera 1993, Pérez-Rodríguez et al. 2007). Total plasma carotenoid concentration (μg/ml) was calculated using a standard curve for lutein (Sigma Chemicals). Lutein was chosen as a reference pigment because previous work has established that this was the main carotenoid circulating in blood of partridges (Blas et al. 2006).
Assessment of immune responsiveness

We used the phytohaemagglutinin (PHA) skin test to measure immune responsiveness on a sample of 22 males. This test consists of an intradermal injection of PHA, which produces a prominent perivascular accumulation of T-lymphocytes followed by macrophage infiltration (Goto et al. 1978). It produces a swelling, the magnitude of which indicates aspects of an individual’s ability to mount a cell-mediated immune response. The response to PHA involves several arms of the immune system (Martin et al. 2006). The immediate response 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 are 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 wing web swelling is likely to measure aspects of both innate and acquired cellular immunity, with a different time course (Kennedy and Nager 2006). The maximum response to PHA is typically 24 h post challenge and the swelling disappears after 48h (Smith et al. 1999). Previous trials showed that the swelling increased 12 h post injection, was maximum at 24 h in our study species, and declined after 36 h (unpubl. data). We measured wing web swelling 24 h after PHA exposure, when it is likely that mainly the first phase of the response is underway and the infiltration by T cells is likely to be a relatively minor, but increasing, component of the cellular response (Martin et al. 2006).

Cell-mediated immune responses can show diurnal variation (Martinez-Padilla 2006). To avoid such bias, we injected all birds 9.00–11.00 am with 0.5 mg of PHA (SIGMA L-8754) suspended in 0.1 ml of physiological saline solution (PBS) at a marked site on the wing web. We measured web thickness at injection site with a pressure-sensitive dial thickness gauge (Mitutoyo Absolute 547) to the nearest 0.01 mm. Web thickness was measured three times prior to injection and 24 hr after injection. Both initial ($R = 0.99$, $F_{23,48} = 510.3$, $P < 0.001$) and final measurements ($R = 0.99$, $F_{23,48} = 336.2$, $P < 0.001$) of wing web thickness were highly repeatable. We calculated the swelling response to PHA as the change at 24 h in average thickness at the injection site.

Parasite abundance estimates

Red-legged partridges are frequently infected by coccidia *Eimeria colchici* and *E. tenella* (Villanúa et al. 2006). We used caecal oocyst counts to estimate coccidia abundance (Mougeot et al. 2004, Villanúa et al. 2006). Early morning, we placed a cardboard sheet under the cages to collect fresh ceacal droppings, because coccidia oocysts are concentrated in ceecal rather than faecal droppings (Villanúa et al. 2006). We collected a sample from 41 males 12TH december 2006. We collected all caecal samples fresh and in the morning (8.00–10.00 am). Samples were stored in individual bags at 4°C to avoid oocyst development and analyzed within four d of collection. For each male, a sub-sample of c. 0.2 g of ceecal material was diluted in 5ml of saturated SO$_4$Zn solution, to allow oocyst flotation, and mixed thoroughly. A sub-sample of this solution was placed in a MacMaster chamber under a microscope in order to count coccidia oocysts (see Mougeot et al. 2004, 2006; Seivwright et al. 2005). We did two counts of oocysts for each sample and used the average count (counts were highly and
significantly repeatable; R = 83, P < 0.001). We used the concentration of coccidia oocysts (per g of caecal sample) as an index of coccidia abundance.

**Statistical analyses**

We used SAS 8.01 for statistical analyses (SAS 2001). Repeatabilities were calculated following Lessells and Boag (1987). We investigated natural correlates among study variables using Generalized Linear Models. We tested whether dependent variables were normally distributed (Wilk Shapiro test, univariate procedure, SAS 2001), and used appropriate transformations when variables were not normally distributed (log-transformation for coccidia oocyst concentration and arcsin transformation for% of eye ring pigmentation). Mean parasite abundance was calculated as a geometric mean (Hudson et al. 2001). When analysing variation in body condition, the dependent variable was log-transformed body mass, with log-transformed tarsus length included as a fixed effect in all models. We used the Princomp procedure (SAS 2001) for the principal component analysis. All tests are two-tailed.

**Results**

Parasites, condition, immune responsiveness and plasma carotenoids

All males were infected by coccidia (geometric mean × SD = 11661 × 1.67; range 77–32,920 oocysts per g; n = 41). Male condition was significantly negatively related to coccidia abundance (F1,40 = 6.29; P = 0.016; slope ± SE: −0.014 ± 0.006) and positively correlated with circulating carotenoids (F1,40 = 9.35; P = 0.004; slope = +0.009 ± 0.003). A greater swelling response to PHA was associated with a better condition (F1,21 = 24.42; P < 0.001; slope = 3.376 ± 0.683), fewer coccidia (F1,21 = 14.98; P < 0.001; slope: −0.096 ± 0.025; Fig. 2a) and more circulating carotenoids (F1,21 = 4.34; P < 0.05; slope: +0.036 ± 0.017). Carotenoid levels were significantly negatively related to coccidia abundance (F1,41 = 4.70; P = 0.036; slope: −0.579 ± 0.267; Fig. 2b).

Ornamentation and plasma carotenoids

Eye ring pigmentation positively correlated with carotenoid levels (F1,39 = 12.36; P = 0.001). We found no relationship between redness of the beak, nostrils and eye rings with circulating carotenoids (beak: F1,39 = 2.23; P = 0.14; nostril: F1,39 = 3.20; P = 0.08; eye ring: F1,39 = 0.90; P = 0.34).

Our PCA analysis on colour traits summarized overall male carotenoid-based ornamentation. The first PC axis, explained 53.7% of variation in ornamental coloration, with eye ring pigmentation, eye ring redness, nostril redness and beak redness all having positive loadings (Table 1). This first axis was thus indicative of overall redness. The second PC axis, explained a further 25.7% of variation, with eye ring coloration and pigmentation having positive loadings, and beak and nostril coloration having negative loadings (Table 1). Carotenoid-based coloration, as summarized by the first principal component, significantly positively correlated with circulating carotenoid levels (F1,39 = 6.86; P = 0.013; slope: +0.180 ± 0.068; Fig. 2c). The second principal component was not significantly related to carotenoid levels (F1,39 = 0.30; P = 0.58).
Table 1. Results of the principal component analysis (Princomp procedure, SAS 2001) on male carotenoid-based ornaments (eye ring pigmentation, eye ring redness, nostril redness and beak redness; see methods).

<table>
<thead>
<tr>
<th>Principal components</th>
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<th>Second</th>
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<tr>
<td>Eye ring pigmentation</td>
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<td>0.529</td>
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<tr>
<td>Eye ring redness</td>
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</tr>
<tr>
<td>Nostril redness</td>
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<tr>
<td>Beak redness</td>
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<td>-0.662</td>
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<tr>
<td>Eigenvalues</td>
<td>2.14</td>
<td>1.03</td>
</tr>
<tr>
<td>Variance explained</td>
<td>- cumulative: 53.7% 79.4% - proportion: 53.7% 25.7%</td>
<td></td>
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</tbody>
</table>

**Ornamentation, condition, parasites and immune responsiveness**

Table 2 summarizes the main predictors of condition, coccidia abundance and swelling response to PHA. Univariate analyses showed that condition was not significantly related to carotenoid-based ornamentation. However, fewer coccidia were predicted from redder eye rings and redder beak, and a greater swelling response to PHA was predicted from redder and more pigmented eye rings (Table 2). We further evaluated the predictive value of overall redness, as summarized by the principal component analysis (Table 1). Male redness (PC1) correlated positively, but not significantly with condition, but predicted coccidia abundance and swelling response to PHA (Table 1).

**Discussion**

**Ornamentation, carotenoids and condition**

Eye ring pigmentation was positively associated with circulating carotenoid levels, but not beak, nostril or eye ring redness. When considering overall redness, as summarized by the first principal component of the PCA on all measured colored traits, redder males were nevertheless those with more circulating carotenoids (Fig. 3), consistent with previous studies on red-legged partridge (Blas et al. 2006) and other birds (reviewed in McGraw 2006) showing that more intense carotenoid pigmentation is associated with more circulating carotenoids.

Ornament expression is expected to be condition-dependent, so that only individuals in prime condition exhibit the most coloured or exaggerated ornaments (Andersson 1994). This is particularly expected from carotenoid-based ornaments because carotenoids cannot be synthesised but must be ingested (Hill and McGraw 2006). Individuals with greater access to food, and in better condition, would ingest more carotenoids and show brighter colour. However, the redness and pigmentation of eye rings and beak of male red-legged partridges was not significantly related to condition. This might be because our study birds were captive and fed ad limitum, and therefore may not have shown enough variation in condition. Previous studies on captive red-legged partridges showed that changes in coloration reflected changes in condition (Blas et al. 2006), or is affected by experimental reductions of condition, via food restriction (Pérez-Rodríguez and Viñuela 2008). Over our range of male condition,
the red coloration better predicted other male qualities, like parasite abundance and immune responsiveness.

**Ornamentation and parasites**

Carotenoid-based signals should be particularly sensitive to parasite infections (Lozano 1994, Hörak et al. 2004, Dawson and Bortolotti 2006, Martinez-Padilla et al. 2007, Mougeot et al. 2007). Overall male redness, and in particular eye ring redness and beak redness predicted well coccidia abundance (Fig. 3). This is consistent with the hypothesis that carotenoid-based ornamentation indicates a male’s current health (parasite infection). Carotenoid availability and trade-offs for the use of available carotenoids for self maintenance and parasite defence versus ornament expression might explain the results. Carotenoid levels were negatively related to coccidia abundance, suggesting that these parasites reduced available carotenoids and ultimately their use for ornamental coloration (Fig. 3). Coccidia might negatively impact on carotenoid levels and carotenoid-based ornamentation in several, non exclusive ways.

After ingestion, carotenoids are incorporated into micelles and enter mucosal cells. The thickening of the gut epithelium caused by coccidiosis has been shown to constrain carotenoid absorption in other birds (Allen 1987, Hörak et al. 2004). Coccidia might thus directly inhibit the uptake of carotenoids and other essential dietary components by damaging the epithelial cells of the host’s intestine.

Coccidia might also mobilise available carotenoids for parasite defence needs, to the detriment of other uses. Because of their molecular composition, carotenoids may act as effective scavengers of free radicals (Burton 1989, Olson 1989, Møller et al. 2000), which are released during immune responses and help to counter invading pathogens (Allen 1997, Halliwell and Gutteridge 1999) and protect against free radicals produced by the immune system (von Schantz et al. 1999). Mounting an immune response to a parasite infection could thus drain carotenoids from the bloodstream (Pérez-Rodriguez et al. 2008) and therefore compromise carotenoid signalling.

Finally, by reducing host condition, coccidia might affect carotenoid transport and metabolism, which are most likely energetically demanding processes (McGraw 2006). A negative impact of coccidia on condition was supported by the negative correlation between condition and coccidia abundance.
Ornamentation and immune responsiveness

Overall redness, and in particular eye ring redness and pigmentation predicted the swelling response to PHA (Fig. 3). This is consistent with studies on other birds showing that carotenoid-based coloration of integuments or beaks indicates the ability of males to respond to a standardized immune challenge (Fairevre et al. 2003a, Peters et al. 2004, Mougeot 2008). In captive birds, carotenoid supplementation via diet has been shown to increase T-cell mediated immune responses (Blount et al. 2003, McGraw and Ardia 2003). Immune activation has also been shown to reduce circulating carotenoids and ultimately ornamentation (Fairevre et al. 2003b, Alonso-Alvarez et al. 2004, Pérez-Rodríguez et al. 2008). Immune responsiveness positively correlated with carotenoid levels, consistent with the hypothesis that more circulated carotenoids allows for greater immune responsiveness.

We found that the swelling response to PHA negatively correlated with coccidia abundance and that parasitized males have fewer circulating carotenoids. Parasites might thus limit immune responsiveness by reducing circulating carotenoids. Coccidia also most likely reduced condition, and immune responsiveness is condition dependent (Alonso-Alvarez and Tella 2001, this study), so parasite effects on condition could also explain the negative relationship between immune responsiveness and parasites. So far, the effect of intestinal parasites on immune responsiveness has been little studied, with inconsistent results (Owen and Clayton 2007). For instance, nematode parasites have been found to reduce immune responsiveness in red junglefowl Gallus Gallus (Johnsen and Zuk 1999) and in red grouse (Mougeot and Redpath 2004). In contrast, intense coccidia multiplication in the host’s digestive tract resulted in the enhancement of the host’s cell-mediated immune function in the greenfinch Carduelis chloris (Saks et al. 2006).

In conclusion, our results support the hypothesis that diseased males produce less red ornaments (Hill and McGraw 2006) and that males with greater carotenoid-based ornamentation handle infections better (Lozano 1994; Dawson and Bortolotti 2006) and are better able to mount an immune response to a novel challenge. Our results support our initial hypothesis that when food is not limited (birds fed ad libitum), the carotenoid-based ornamentation of males reflects their current health rather than condition. Under natural conditions, food is often limited, so future works should investigate the relative importance of foraging ability versus health as limiting factors of ornament expression. Interestingly, experimental food restriction (reduction in condition) rapidly reduced the eye ring pigmentation but not the eye ring redness or bill redness of red-legged partridges (Pérez-Rodríguez and Viñuela 2008), which we found to be good indicators of coccidia infection and immune responsiveness. Thus, it is possible that the information content of different ornaments combine to reliably indicate both condition and health. Future studies should investigate experimentally the relative effect of condition (using food restrictions) and parasites (using deparasitation and re-infections) on the expression of ornaments.

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