Nematode parasites reduce carotenoid-based signalling in male red grouse

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Carotenoid-based signals should be particularly sensitive to parasites (Lozano 1994), though experimental evidence remains limited (e.g. Hill & McGraw 2006). For instance, coccidia can directly reduce carotenoid uptake (Hörak et al. 2004), and cestodes might negatively influence carotenoid signalling (Figueroa et al. 2005). Nematodes are common intestinal parasites of vertebrates, and often have profound effects on hosts (Wakelin 1978); however, their effects on circulating carotenoids and carotenoid-dependent ornamentation have never been tested experimentally.

We manipulated nematode parasites in male red grouse and investigated the effects on carotenoid-based signalling. Red grouse display red supra-orbital combs pigmented by carotenoids (Mougeot et al. 2007) that function in intra- and inter-sexual selection (Mougeot et al. 2004, 2007). Using an anthelmintic drug, we reduced infection by Trichostrongylus tenuis worms. This main parasite of red grouse negatively impacts condition, productivity and survival (Hudson 1986). We predicted a reduction in T. tenuis would increase plasma carotenoids and the pigmentation of grouse combs.

2. MATERIAL AND METHODS

(a) Experiment

In autumn 2005 (16 October–1 November), we caught 37 males on Edinglassie Estate, northeast Scotland (57°12′N–3°07′W). Each was ringed, fitted with a radio collar (TW3-necklace tag, Biotrack) and aged (young, i.e. hatched that summer or old). Males were randomly assigned to one of two treatments: dosed (parasite reduction) or control. After collecting faecal samples for parasite counts, control males were given 1 ml oral dose of water, and dosed males were given 1 ml of anthelmintic (levamisole hydrochloride, Nilverm Gold), a drug effective at reducing T. tenuis worms. This main parasite of red grouse would increase plasma carotenoids and the pigmentation of grouse combs.

(b) Comb redness

High-resolution (2272×1704 pixels) pictures of the flattened comb were taken at a standard distance (50 cm) using the flash of the digital camera (Nikon Coolpix 4500). The same grey reference chip was placed beside the comb for each picture. We analysed digital images using Adobe Photoshop v. 7.0, measuring the average component of red (R) from the largest continuous area within the combs and the grey reference using the RGB system (see electronic supplementary material). Comb redness measures were highly repeatable (see electronic supplementary material).

(c) Plasma carotenoid concentration

Carotenoids were quantified by diluting 60 μl of plasma in acetone (1:10). The mixture was vortexed and centrifuged at 10 000 r.p.m. for 10 min. The supernatant was examined in a ShimadzuUV-1603 spectrophotometer and we determined the optical density at 446 nm, the wavelength of maximal absorbance for lutein (Minguez-Mosquera 1993), the most common circulating carotenoid in birds (Hill & McGraw 2006). This wavelength has been considered as a reliable index of total carotenoids (Blount et al. 2003; McGraw et al. 2003). Plasma carotenoid concentration (μg ml−1) was calculated using a standard curve of lutein (Sigma Chemicals).

(d) Parasite abundance

We used faecal egg concentrations to estimate coccidia and T. tenuis abundance. Samples were stored at 4°C to inhibit egg development and analysed within 5 days of collection to ensure reliable estimates (Seiwright et al. 2004, see electronic supplementary material).

(e) Statistical analyses

We used SAS v. 9.1. Counts of coccidia eggs and T. tenuis worms were fitted to generalized linear mixed models (GLMMs) using a Poisson error distribution. Plasma carotenoid concentration and comb redness were fitted to GLMMs using a normal distribution.

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3. RESULTS

Before treatment, comb redness positively correlated with circulating carotenoids \((F_{2,13}=3.17, \ p=0.084); \) significantly so when parasites were included as covariates \((F_{1,129}=6.67, \ p=0.015; \) Trichostrongylus tenuis, \(p=0.011\) and coccidia, \(F_{1,129}=2.29, \ p=0.141\)). Circulating carotenoids tended to decrease with increasing coccidia and T. tenuis intensities \((F_{1,31}=3.41, \ p=0.074\) and \(F_{1,31}=2.53, \ p=0.122\), respectively), significantly so when comb redness was a covariate (coccidia, \(F_{1,29}=6.13, \ p=0.019\) and T. tenuis, \(F_{1,29}=4.99, \ p=0.033\); redness, \(F_{1,29}=6.67, \ p=0.015\); figure 1). Comb redness correlated negatively with T. tenuis abundance \((F_{1,29}=7.49, \ p=0.010; \) figure 1b). These relationships did not differ between age groups (all \(p>0.38\).

Before treatment, T. tenuis and coccidia prevalences were 89.2 and 100\%, respectively. Old males had more T. tenuis than young grouse (table 1).

Trichostrongylus tenuis abundance decreased significantly more in dosed than in control males (table 1; figure 2a; see also table S1 in electronic supplementary material) independently of bird age. Coccidia abundance was not affected by treatment (table 1). Young males had more coccidia than old males before treatment, but changes over time in abundance did not differ between treatment groups (table 1), in both old and young birds.

Prior to treatment, plasma carotenoid concentration did not differ between treatment and age groups (both \(p>0.46\)). Circulating carotenoids increased significantly more in dosed than in control birds (table 1, figure 2b), in both young and old males. Comb redness also increased significantly more in dosed than in control birds (table 1, figure 2c and electronic supplementary material) in both young and old males. Coccidia abundance did not influence redness after controlling for treatment effects \((F_{1,17}=0.29, \ p=0.669)\).

4. DISCUSSION

In untreated males, comb redness increased with circulating carotenoids, significantly so when parasites were taken into account. Thus, the relationship between ornament coloration and circulating carotenoids, which has been found in several other species...
Our treatment was effective at reducing *T. tenuis* worms, increasing circulating carotenoids and ultimately enhancing ornamental coloration. It is known that other intestinal parasites, particularly coccidia (McGraw & Hill 2000; Hörak et al. 2004) influence carotenoid-based signals in captive birds. Our anthelmintic treatment reduced nematode infection without significantly affecting coccidia parasites. Our experimental results were also consistent with the correlative results, and both indicated that *T. tenuis* nematodes reduce circulating carotenoids and redness of the comb. We are thus confident that our results indicate a negative effect of nematodes on plasma carotenoids and on carotenoid-based ornamentation. Despite a high prevalence, *T. tenuis* abundance was low in our study, compared with the range observed in red grouse (up to 30 000 worms, Hudson 1986). Thus, even subtle variations in nematode infection can affect ornamentation.

Nematodes can affect carotenoid signals in several, non-exclusive ways. The thickening of the gut epithelium caused by coccidiosis has been shown to constrain carotenoid absorption (Allen 1987). Adult nematodes inhabit the caeca of red grouse (Seiveright et al. 2004) and cause significant damage to epithelial tissues. Grouse have particularly long caeca to maximize digestion and absorption of plant nutrients. Although we do not know if carotenoid absorption takes place in the caeca, the caecal damage caused by *T. tenuis* worms could constrain absorption and explain the negative effect of nematodes on circulating carotenoids. *Trichostrongylus tenuis* worms might also reduce the production of high-density lipoproteins and their incorporation into ornaments (McGraw et al. 2006) or directly compete with the bird for carotenoids (Mawson & Wakabongo 2002). Finally, nematodes can also have other systemic effects on carotenoid availability (Hill et al. 2004) as carotenoids may be diverted to boost the immune system against nematodes instead of being displayed in ornaments (Møller et al. 2000; Blount et al. 2003).

Nematodes are among the commonest parasites of vertebrates (Wakelin 1978), and have the potential to reduce plasma carotenoid availability and carotenoid use for ornamentation, as demonstrated by our experiment. This should stimulate more experiments on wild and captive animals, and more detailed investigation of the mechanisms by which nematode parasites influence carotenoid signals.

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ELECTRONIC SUPPLEMENTARY MATERIAL (ESM)

Comb colour measurements

Figure S1 shows a representative digital photograph taken of the red grouse combs. The areas used for measuring the colour of the comb and grey reference are shown. We used the largest rectangular area fitted within the comb area, as shown in the figure. The grey reference was obtained from Dulux® (Ebony Mists 1–6) grid. The same reference (Ebony Mists 4) was used for all comb photographs. We obtained the average component of red (R) of the combs and from the grey reference for each comb, using RGB system and Adobe Photoshop 7.0.

![Figure S1. Digital photograph of a male red grouse showing the area of comb and grey reference used for colour measurements.](image)

We evaluated the repeatability by comb colour measures by measuring twice a sample of males in two ways: repeatability of R-values (adjusted for the R-value of the reference) (1) within the same picture and (2) between two different pictures of the
same combs taken at the same time. Repeatability values were calculated following Lessells and Boag (1987). Comb redness measurement were highly and significantly repeatable both within (repeatability = 0.98, $F_{14,29} = 111.09$, $p < 0.001$) and between pictures (repeatability = 0.80, $F_{13,27} = 8.98$, $p < 0.001$)

**Parasite counts**

We used faecal eggs counts to estimate the abundance of *T. tenuis* and coccidian in free living males, following a reliable and well established method for red grouse (Shaw & Moss, 1989; Moss et al., 1993; Mougeot et al., 2003; Seivwright et al., 2005; Mougeot et al., 2005; Mougeot et al., 2006).

We used a McMaster egg counting technique (MAFF, 1986). For each faecal sample, approximately 0.20 g (range 0.19–0.21 g) of weighed mixed faecal material was put into a shaker tube with 10 glass balls and 5ml of saturated NaCl solution. The tube was shaken until the faecal matter was suspended. Using a Pasteur pipette, a sample of the faecal suspension was extracted and carefully run into one chamber of a McMaster counting slide. The tube was shaken again and another sample extracted and run into the second section of the chamber. The saline suspension was left to settle for 2–3 min, allowing the eggs to float to the top of each chamber. Eggs were then counted beneath a marked grid on each chamber using a compound microscope with 40× magnification. Two separate counts of coccidia and *T. tenuis* eggs were performed for each sample. Counts of both parasites’ eggs were highly and significantly repeatable (*T. Tenuis* egg counts: repeatability = 0.97; $F_{24,49} = 68.44$, $p < 0.001$; Coccidia egg counts: repeatability = 0.83, $F_{24,49} = 10.55$, $p < 0.001$). The number of eggs per gram of faecal material was calculated by multiplying the average number of eggs counted under both grids by the total volume of faecal suspension contained in both chambers and then dividing this by the quantity of faeces used in the suspension.
### Additional results

**Table S1.** Mean (± SD) abundance of coccidia (oocysts per g) and *T. tenuis* (worms per grouse), plasma carotenoid concentration (µg/ml), measured relative to a lutein standard, and comb redness before and after treatment in both experimental groups.

<table>
<thead>
<tr>
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<th>Coccidia abundance</th>
<th>T. tenuis abundance</th>
<th>Carotenoids</th>
<th>Comb redness</th>
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<tr>
<td></td>
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</table>
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