

Nematode parasites reduce carotenoid-based signalling in male red grouse

Jesús Martínez-Padilla^{1,*}, François Mougeot^{1,2,3}, Lorenzo Pérez-Rodríguez² and Gary R. Bortolotti⁴

¹Centre for Ecology and Hydrology-Banchory, Banchory, Hill of Brathens, Aberdeenshire AB31 4BW, UK

²Instituto de Investigación en Recursos Cinegéticos, CSIC-UCLM-JCCM, Ronda de Toledo s/n, 13071 Ciudad Real, Spain

³School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK

⁴Department of Biology, University of Saskatchewan, Saskatoon, Canada S7N 5E2

*Author for correspondence (jmart@ceh.ac.uk).

Carotenoids determine the yellow–red colours of many ornaments, which often function as signals of quality. Carotenoid-based signalling may reliably advertise health and should be particularly sensitive to parasite infections. Nematodes are among the commonest parasites of vertebrates, with well-documented negative effects on their hosts. However, to date, little is known about the effects that these parasites may have on carotenoid-based signalling. Tetraonid birds (grouse) exhibit supra-orbital combs, which are bright integumentary ornaments pigmented by carotenoids. We tested the effect of the nematode parasite *Trichostrongylus tenuis* on signalling in free-living male red grouse *Lagopus lagopus scoticus*. We show that experimentally reduced nematode infection increases plasma carotenoid concentration and comb redness, demonstrating for the first time that nematodes can influence carotenoid-based signals.

Keywords: carotenoids; comb; nematodes; red grouse *Lagopus lagopus scoticus*; *Trichostrongylus tenuis*

1. INTRODUCTION

The brightly coloured ornaments of animals often function as reliable signals of quality, indicating better body condition or ability to resist parasites (Hamilton & Zuk 1982). Carotenoid-based signals are among the most familiar criteria for mate choice (Hill & McGraw 2006). Identifying limiting factors along the pathway from nutritional access to coloration is essential for the understanding of how carotenoid-based ornaments have evolved and are maintained as honest signals (Hill & McGraw 2006). Vertebrates cannot produce carotenoids, so carotenoid intake can limit ornament expression (Olson & Owens 1998). Carotenoids also have beneficial physiological functions, being immunostimulants and antioxidants (Møller *et al.* 2000). The trade-offs resulting from carotenoid use in self-maintenance versus ornamentation may further confer honesty (Faivre *et al.* 2003).

Electronic supplementary material is available at <http://dx.doi.org/10.1098/rsbl.2006.0593> or via <http://www.journals.royalsoc.ac.uk>.

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 (Figuerola *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* (Hudson 1986). We recaptured 30 males 18 ± 4 days after treatment. Time between capture and recapture did not differ between groups (general linear model, $F_{1,26}=0.22$, $p=0.643$). At each capture, we took a blood sample from the wing vein and a digital photograph of the comb. Blood was centrifuged and plasma kept frozen at –20°C. Males were kept overnight in individual boxes to collect faecal samples.

(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 Shimadzu UV-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

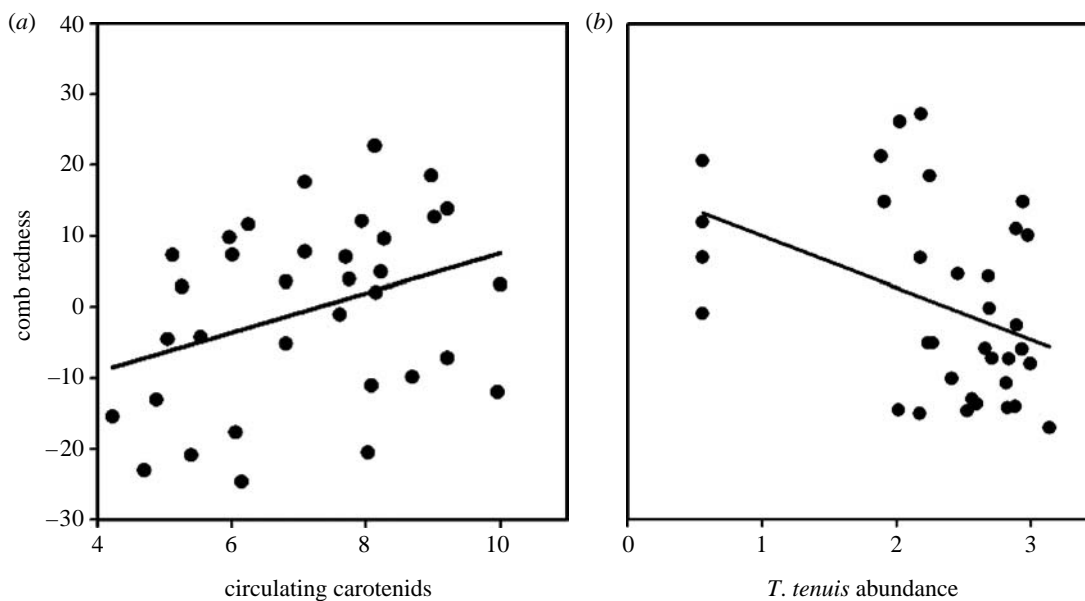


Figure 1. Relationships between comb redness and (a) plasma carotenoid concentration ($\mu\text{g ml}^{-1}$). (b) *T. tenuis* abundance (worms per grouse) before treatment.

Table 1. Effects of age, treatment and recapture on coccidia and *T. tenuis* abundance, plasma carotenoid concentration and comb redness.

dependent variables	coccidia abundance ^a			<i>T. tenuis</i> worm abundance ^a			carotenoids			comb redness ^b		
	d.f.	<i>F</i>	<i>p</i>	d.f.	<i>F</i>	<i>p</i>	d.f.	<i>F</i>	<i>p</i>	d.f.	<i>F</i>	<i>p</i>
age (<i>A</i>)	1,34.1	4.67	0.038	1,31.8	54.79	<0.001	1,24	0.47	0.498	1,24	0.02	0.879
treatment (<i>T</i>)	1,33.7	0.50	0.486	1,26.8	5.28	0.030	1,24	0.18	0.675	1,24	0.04	0.842
recapture (<i>R</i>)	1,32.5	5.75	0.022	1,29.1	7.45	0.011	1,24	33.23	<0.001	1,24	2.51	0.126
<i>T</i> × <i>R</i>	1,33.8	0.17	0.686	1,15.6	8.59	0.010	1,24	4.37	0.046	1,24	14.50	0.019
<i>A</i> × <i>T</i> × <i>R</i>	4,17.3	0.24	0.835	4,15.4	1.94	0.311	4,15	1.71	0.200	4,21	1.12	0.372

^a GLMM models were performed with Poisson error and log link function.

^b For analyses of comb redness, all models including the *R*-value of the grey reference as a covariate ($p < 0.001$).

(Shapiro–Wilk tests, NS). When testing for treatment effects, we included ‘individual’ as a random effect, to account for repeated measures. We tested for differences between treatment groups in changes over time of variables by including ‘recapture’ (before versus after treatment), treatment (dosed versus control), age (old and young) and their interactions as fixed effects. *Trichostrongylus tenuis* and coccidia abundances were log transformed when included as explanatory variables. For analyses of comb redness, *R*-values were standardized using *R*-values from the grey reference, included as a covariate in all models. All tests were two-tailed.

3. RESULTS

Before treatment, comb redness positively correlated with circulating carotenoids ($F_{2,33} = 3.17$, $p = 0.084$; figure 1a), significantly so when parasites were included as covariates ($F_{1,29} = 6.67$, $p = 0.015$; *T. tenuis*, $p = 0.011$ and coccidia, $F_{1,29} = 2.29$, $p = 0.141$). 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

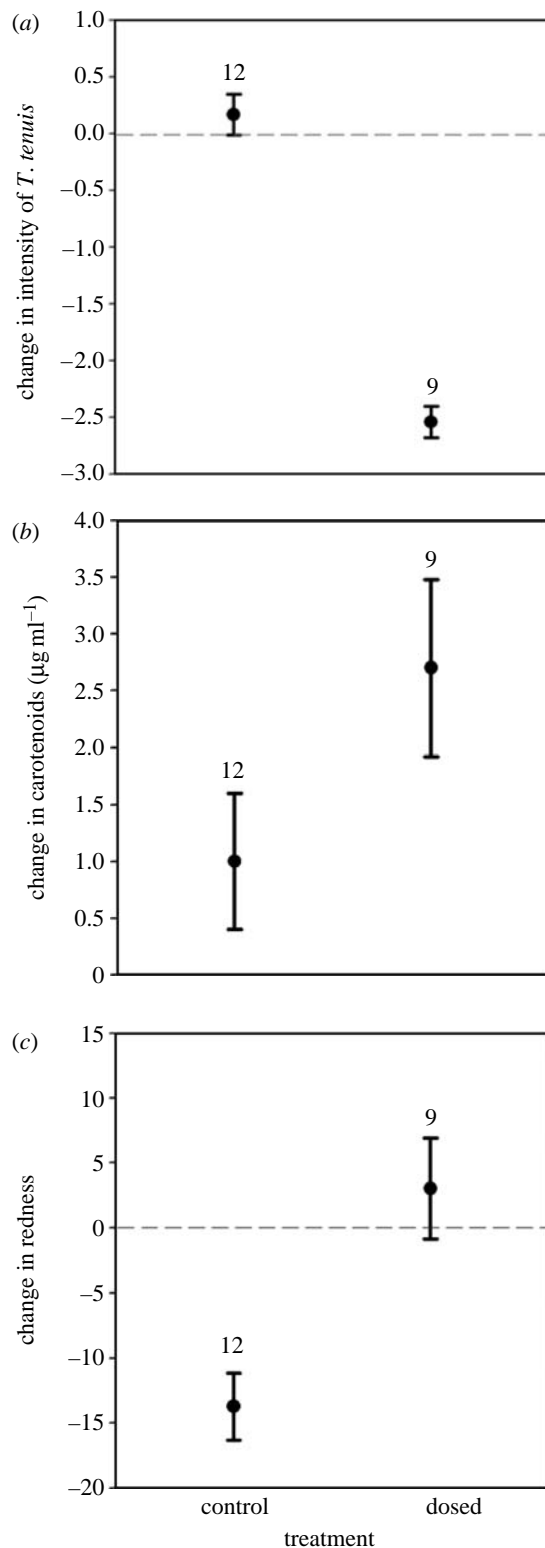


Figure 2. Mean (\pm s.d.) changes in (a) *T. tenuis* abundance (log transformed), (b) plasma carotenoid concentration and (c) comb redness in control and dosed males. Sample sizes above bars are shown.

(Hill & McGraw 2006), was only revealed when parasites provided the appropriate context. Similarly, the negative relationships between carotenoid levels and parasites were better understood when comb colour was taken into account. The negative relationships between both coccidia and *T. tenuis* parasites and carotenoid levels suggested that both parasites limited carotenoid-based signalling.

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; Hřrak 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 (Seivwright 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 (Mřller 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.

We thank the landowner and D. Calder from Edinglassie Estate for allowing us to conduct the experiment. J.M.P., F.M. and L.P.R. were supported by post- and pre-doctoral grants (Ministerio de Educaci3n y Ciencia, Spain). J. Vicente, J. Blount and R. Moss improved greatly an early version of the manuscript. GRB was supported by NSERC.

Allen, P. C. 1987 Physiological response of chicken gut tissue to coccidial infection: comparative effects of *Eimeria acervulina* and *Eimeria mitis* on mucosal mass, carotenoid content, and brush border enzyme activity. *Poult. Sci.* **66**, 1306–1315.

- Blount, J. D., Metcalfe, N. B., Birkhead, T. R. & Surai, P. F. 2003 Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* **300**, 125–127. (doi:10.1126/science.1082142)
- Faivre, B., Gregoire, A., Preault, M., Cezilly, F. & Sorci, G. 2003 Immune activation rapidly mirrored in a secondary sexual trait. *Science* **300**, 103. (doi:10.1126/science.1081802)
- Figuerola, J., Torres, J., Garrido, J., Green, A. J. & Negro, J. J. 2005 Do carotenoids and spleen size vary with helminth load in greylag geese? *Can. J. Zool.* **83**, 389–395. (doi:10.1139/z05-022)
- Hamilton, W. D. & Zuk, M. 1982 Heritable true fitness and bright birds: a role for parasites? *Science* **218**, 384–387. (doi:10.1126/science.7123238)
- Hill, G. E. & McGraw, K. J. 2006 *Bird coloration. Mechanisms and measurements*. Cambridge, MA: Harvard University Press.
- Hill, G. E., Farmer, K. L. & Beck, M. L. 2004 The effect of mycoplasmosis on carotenoid plumage coloration in male house finches. *J. Exp. Biol.* **207**, 2095–2099. (doi:10.1242/jeb.00998)
- Hörak, P., Saks, L., Karu, U., Ots, I., Surai, P. F. & McGraw, K. J. 2004 How coccidian parasites affect health and appearance of greenfinches. *J. Anim. Ecol.* **73**, 935–947. (doi:10.1111/j.0021-8790.2004.00870.x)
- Hudson, P. J. 1986 *The red grouse: the biology and management of a wild gamebird*. Fordingbridge, UK: The Game Conservancy Trust.
- Lozano, G. A. 1994 Carotenoids, parasites, and sexual selection. *Oikos* **70**, 309–311.
- Mawson, A. R. & Wakabongo, M. 2002 Onchocerciasis-associated morbidity: hypothesis. *Trans. R. Soc. Trop. Med. Hyg.* **96**, 541–542. (doi:10.1016/S0035-9203(02)90434-7)
- McGraw, K. J. & Hill, G. E. 2000 Differential effects of endoparasitism on the expression of the carotenoid- and melanin-based ornamental coloration. *Proc. R. Soc. B* **267**, 1525–1531. (doi:10.1098/rspb.2000.1174)
- McGraw, K. J., Gregory, A. J., Parker, R. S. & Adkins-Regan, E. 2003 Diet, plasma carotenoids, and sexual coloration in the zebra finch (*Taeniopygia guttata*). *Auk* **120**, 400–410. (doi:10.1642/0004-8038(2003)120[0400:DPCASC]2.0.CO;2)
- McGraw, K. J., Correa, S. M. & Adkins-Regan, E. 2006 Testosterone upregulates lipoprotein status to control sexual attractiveness in a colorful songbird. *Behav. Ecol. Soc.* **60**, 117–122. (doi:10.1007/s00265-005-0135-3)
- Mínguez-Mosquera, I. 1993 *Clorofilas y carotenoides en tecnología de alimentos*. Sevilla, Spain: Universidad de Sevilla.
- Møller, A. P., Biard, C., Blount, J. D., Houston, D. C., Ninni, P., Saino, N. & Surai, P. F. 2000 Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? *Avian Poult. Biol. Rev.* **11**, 137–159.
- Mougeot, F., Irvine, J. R., Seivwright, L., Redpath, S. M. & Piertney, S. 2004 Testosterone, immunocompetence, and honest sexual signaling in male red grouse. *Behav. Ecol.* **15**, 930–937. (doi:10.1093/beheco/arih087)
- Mougeot, F., Martínez-Padilla, J., Pérez-Rodríguez, L. & Bortolotti, G. R. 2007 Carotenoid-based coloration and ultraviolet reflectance of the sexual ornaments of grouse. *Behav. Ecol. Soc.* (doi:10.1007/s00265-006-0304-z)
- Olson, V. A. & Owens, I. P. F. 1998 Costly sexual signals: are carotenoids rare, risky or required? *Trends Ecol. Evol.* **13**, 510–514. (doi:10.1016/S0169-5347(98)01484-0)
- Seivwright, L., Redpath, S. M., Mougeot, F., Watt, L. & Hudson, P. J. 2004 Faecal egg counts provide a reliable measure of *Trichostrongylus tenuis* intensities in free-living red grouse *Lagopus lagopus scoticus*. *J. Helminthol.* **78**, 69–76. (doi:10.1079/JOH2003220)
- Wakelin, D. 1978 Immunity to intestinal parasites. *Nature* **273**, 617–620. (doi:10.1038/273617a0)

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.

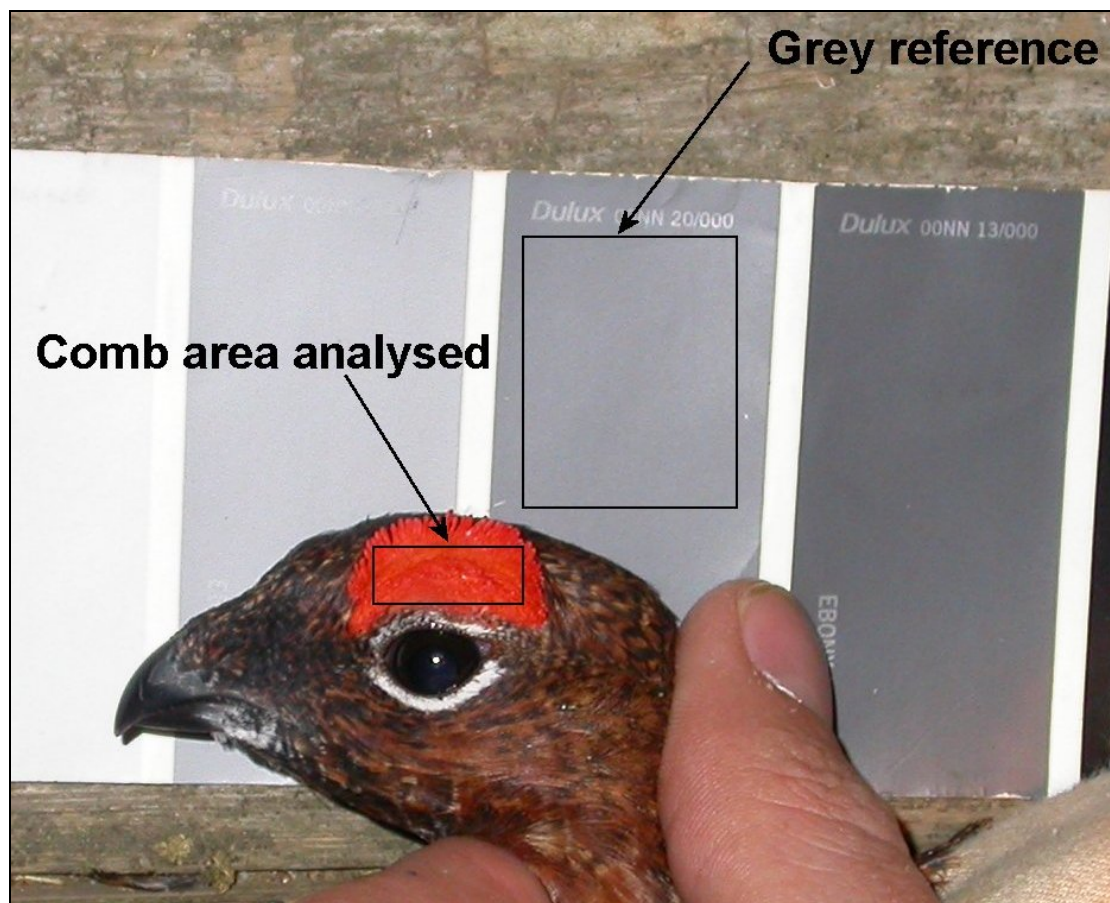


Figure S1. Digital photograph of a male red grouse showing the area of comb and grey reference used for colour measurements.

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 (\pm SD) abundance of coccidia (oocysts per g) and *T. tenuis* (worms per grouse), plasma carotenoid concentration ($\mu\text{g/ml}$), measured relative to a lutein standard, and comb redness before and after treatment in both experimental groups.

		Coccidia abundance			<i>T.tenuis</i> abundance			Carotenoids			Comb redness		
		mean	sd	n	mean	sd	n	mean	sd	n	mean	sd	n
Control	Before treatment	9182.91	10612.03	25	446.28	376.03	25	12.21	1.59	25	33.26	2.77	25
	After treatment	5654.94	6780.33	21	372.67	241.70	21	13.21	1.82	21	19.53	2.98	21
Dosed	Before treatment	10939.63	14885.68	12	352.61	290.73	12	11.40	1.50	12	25.71	4.22	12
	After treatment	4881.33	4496.69	9	0.00	0.00	9	14.10	1.44	9	28.73	4.67	9

References

Lessells CM and Boag PT, 1987 Unrepeatable repeatabilities: a common mistake. *Auk* 104, 116-121

MAFF 1986 *Manual of veterinary parasitological laboratory techniques*. Ministry of Agriculture, Fisheries and Food.

Moss R, Watson A, Trenholm IB, Parr R, 1993 Cecal threadworms *Trichostrongylus tenuis* in Red grouse *Lagopus lagopus scoticus* - Effects of weather and host density upon estimated worm burdens. *Parasitology* 107, 199-209

Mougeot F, Evans SA, Redpath SM, 2005 Interactions between population processes in a cyclic species: parasites reduce autumn territorial behaviour of male red grouse. *Oecologia* 144, 289-298

Mougeot F, Martínez-Padilla J, Pérez-Rodríguez L, Bortolotti GR, 2006 Carotenoid-based coloration and ultraviolet reflectance of the sexual ornaments of grouse. *Behav. Ecol. Soc.*

Mougeot F, Redpath SM, Leckie F, Hudson PJ, 2003 The effect of aggressiveness on the population dynamics of a territorial bird. *Nature* 421, 737-739

Seiwright LJ, Redpath SM, Mougeot F, Leckie F, Hudson PJ, 2005 Interactions between intrinsic and extrinsic mechanisms in a cyclic species: testosterone increases parasite infection in red grouse. *Proc R Soc B* 272, 2299-2304

Shaw JL and Moss R, 1989 Factors affecting the establishment of the cecal threadworm *Trichostrongylus tenuis* in Red grouse (*Lagopus lagopus scoticus*). *Parasitology* 99, 259-264