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How coccidian parasites affect health and appearance of greenfinches

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Summary

1. The aim of this study was to examine the mechanisms by which parasites can affect the expression of ornamental traits.
2. Levels of an intestinal coccidian parasite, *Isospora lacazei*, were manipulated in captive male greenfinches (*Carduelis chloris*) by suppressing the natural infections with a coccidiostatic sulphonamide drug. Subsequently, half the birds were experimentally infected, while another half continued receiving medication.
3. Over the course of the experiment the effect of our treatments upon 14 mainly haemato-serological condition indices was recorded. Additionally, changes in colour and carotenoid content of yellow tail and breast feathers, which serve as sexually dimorphic ornamental traits, were measured.
4. Eighty-nine per cent of birds hosted chronic isosporan infection before the experiment, yet experimental inoculation with mixed parasite strains resulted in drastic but transient decreases in serum carotenoid, vitamin E, triglyceride and albumin concentrations, and reduced body mass, indicating serious pathology and probable nutrient malabsorption due to damaged intestinal epithelium.
5. Laboratory-grown tail feathers of infected birds contained 52% less carotenoids and also had smaller values of chroma and hue than those of medicated birds.
6. These results suggest that coccidian infection reduced the expression of plumage coloration by creating a deficiency of carotenoids available for deposition in ornamental feathers.

Key-words: carotenoids, coccidia, experimental infection, plumage colour, vitamin E.

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Introduction

Secondary sexual characters, such as the bright plumages of many bird species, are presumed to have evolved as signals of individual quality (Zahavi 1975). If the expression of such characters is sensitive to the health status of the bearer, individuals can signal their abilities to provide direct and/or genetic benefits to their prospective mates. The concept of parasite-mediated sexual selection proposes that only individuals most resistant to infections can express elaborate ornamentation because parasites withdraw resources that their hosts could otherwise invest into sexual display (Hamilton & Zuk 1982). Recently, it has been proposed that expression of different types of ornamental traits is suppressed by oxidative stress, generated in the process of extensive production of free radicals during immune responses (von Schantz *et al*. 1999). Among such traits, bright carotenoid-based integumental colours have been considered particularly sensitive to oxidative stress and host infection status (e.g. Lozano 1994).

Carotenoids participate in immuno-regulation and -stimulation, lymphocyte proliferation, free-radical scavenging and detoxification (review in Møller *et al*. 2000). Because animals cannot synthesize carotenoids (and hence have to acquire them from food), a trade-off

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between investment of carotenoids in maintenance and ornamentation can be predicted. Individuals that are forced to fight infections during the formation of carotenoid-based ornaments are expected to have less carotenoids available for developing colourful traits. (e.g. Lozano 1994; Olson & Owens 1998; Møller *et al*. 2000). However, despite the accumulating evidence on carotenoid-based sexual signalling, the exact mechanisms generating trade-offs between individual health status and signal expression are not completely understood (e.g. Olson & Owens 1998; Hill 1999). Therefore, to interpret the increasing number of correlative studies about the relationships between ornamental traits and parasite loads (reviewed in Møller *et al*. 2000), a detailed knowledge about the physiological processes underlying the effect of parasitism upon host health and appearance is needed. A valuable way to obtain such knowledge is by manipulating host infection status (through experimental infection and/or medication) and simultaneously monitoring the hosts' physiological condition and development of ornamental traits.

The aim of this study is to analyse the effect of coccidian infection upon health state and signal-trait expression in male greenfinches [*Carduelis chloris* (Linnaeus)]. Intestinal coccidians from the genus *Isospora* (Protozoa, Apicomplexa) infect a number of songbird species in the wild (reviewed by Giacomo *et al*. 1997; Duszynski, Couch & Upton 2000; McGraw & Hill 2000). Related species (coccidians from the genus *Eimeria*) are common parasites of poultry where they directly inhibit the uptake of essential dietary components, including carotenoids in the gastrointestinal tract of chickens (e.g. Allen 1987, 1997; Allen & Fetterer 2002a,b) and consequently depress carotenoid-based pigmentation of skin and legs ('pale bird syndrome'; Tyczkowski, Schaeffer & Hamilton 1991). Published evidence about the effects of coccidians upon carotenoid-based ornaments in wild species is more scarce since only two studies in cardueline finches have demonstrated that experimental infection with *Isospora* sp. causes birds to develop less saturated carotenoid-based plumage and bill coloration (Brawner, Hill & Sundermann 2000; McGraw & Hill 2000).

Greenfinches are medium-sized (*c*. 28 g) gregarious seed-eating passerines native to the western Palearctic region. Males are larger and more colourful than females, with old males developing olive-green plumage on the back, bright or greenish yellow colour in the breast, and striking bright yellow markings on the primaries, primary coverts and sides of the tail feathers. Females are more olive-brown and yellowish-buff, having faint brown streaks on back and lacking full yellow tints in their plumage (Cramp & Perrins 1994). The male plumage brightness (measured by visual scoring) has been shown to be a sexually selected trait, as more brightly coloured male greenfinches tend to be favoured by females as mates (Eley 1991). It has also been shown that males with more yellow ornamental feathers are less likely to be heavily infected with haemoparasites (Merilä, Sheldon & Lindström 1999) and have higher virus clearance rates (Lindström & Lundström 2000). The yellow pigments in the plumage of greenfinches consist of carotenoids, such as the canary xanthophylls (Stradi *et al*. 1995; Saks, McGraw & Hõrak 2003).

To describe the physiological processes accompanying coccidian infection in greenfinches, we applied various clinical screening tests including (i) estimates for total and differential leucocyte counts, (ii) serum protein concentrations and profiles, (iii) serum triglyceride concentration, (iv) serum concentrations of fat-soluble antioxidants such as carotenoids and vitamins E and A and (v) body mass. To estimate the effect of infections upon ornamental traits, we measured spectrophotometrically the colour of yellow tail and breast feathers and the carotenoid content of tail feathers. To our knowledge, this is the first time that such a diverse array of physiological and colourimetric measures have been used to estimate the impact of parasitism upon health and appearance in a wild animal species.

Materials and methods

A total of 28 male greenfinches were caught in mist-nets in the Vaibla bird station in Central Estonia (58°24′ N; 26°3′ E) between 2 and 20 October 2002. Birds were transported to Tartu and housed in individual indoor cages (27 × 51 × 55 cm). The study was carried out from 3 January (day 1) to 6 March (day 63). The birds were fed *ad libitum* with sunflower seeds and tap water. Sand and boiled chicken eggshell mixture was provided to the birds during a 1-week period (days 33–40 of experiment). During the study, birds were held on the natural day-length cycle. The study was conducted under the license from the Estonian Ministry of the Environment and the birds were released into their natural environment after the experiment.

RESEARCH PROTOCOL

On the morning (0700–0930 h) of day 1, all birds were blood-sampled to obtain individual, pre-experimental haemato-serological measurements (first time point in Figs 1 and 2). Two weeks after the first sampling, all the birds were treated against coccidia for 5 days (days 15–19) by adding Vetacox PLV (Sanofy-Synthelabo Inc., Paris, France) to the birds' drinking water (1 g of Vetacox dissolved in 2 L of water). Vetacox, like any other sulphonamide drug, is a broad-spectrum antimicrobial which is efficient against aerobic bacteria and protozoa. In microbial cells, sulphonamides interfere with the biosynthesis of folic acid (required for DNA synthesis) and thereby prevent multiplication of microbes (e.g. Appelgate 1983). Vetacox does not contain carotenoids or any other antioxidants, nor is there any reason to believe that it would interfere with carotenoid metabolism. We therefore assumed that all differences between medicated and infected birds arise entirely due to the antimicrobial action of the drug, rather than due

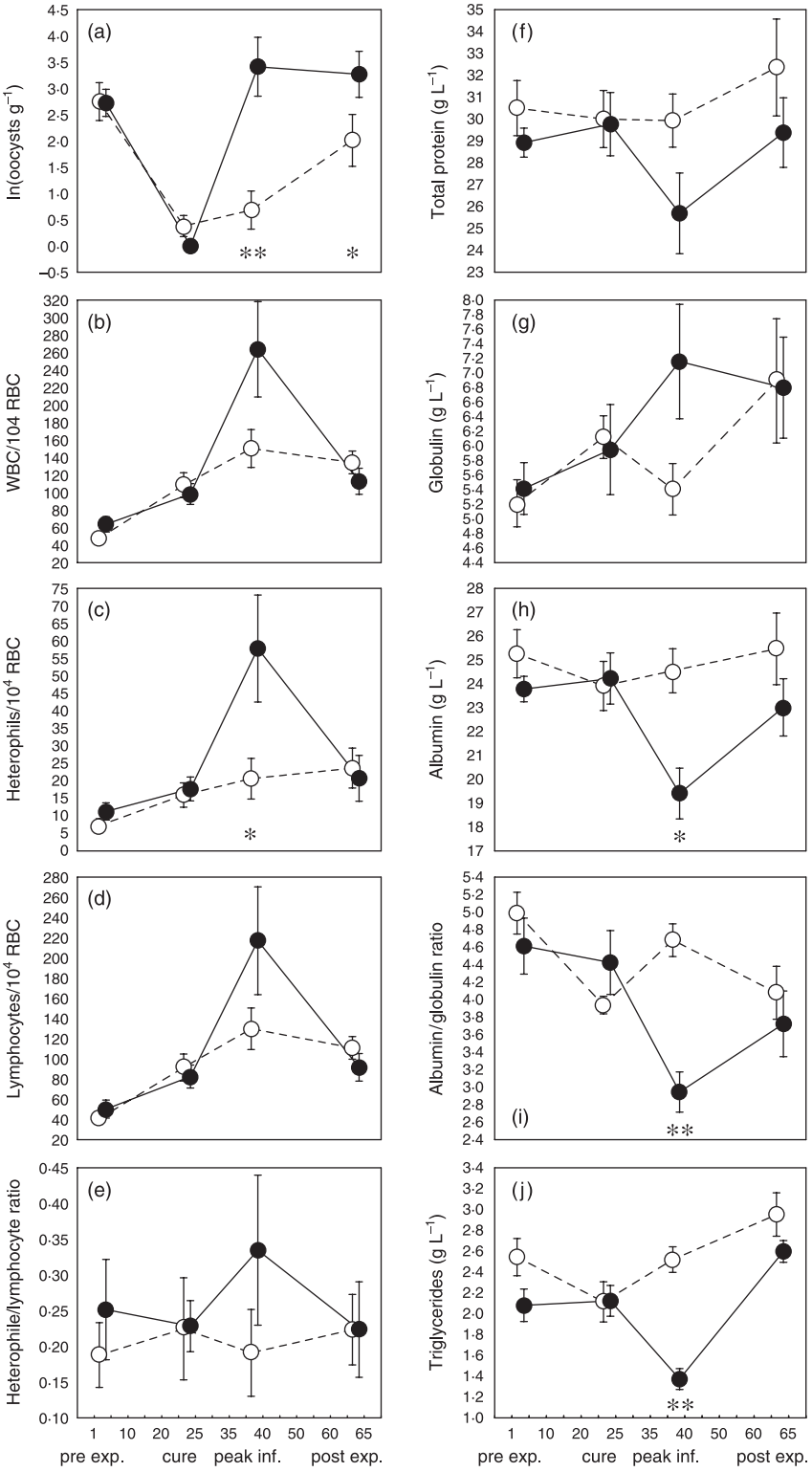

Fig. 1. Effect of manipulation of parasite load upon condition indices of greenfinches. Filled circles, infected group; empty circles, medicated group. Asterisks denote significance of pairwise contrasts between infected and medicated group (*$P < 0{\cdot}05$; **$P < 0{\cdot}001$). The course of experiment in days is noted in *x*-axes of the lower graphs. Sample sizes and statistics are given in Tables 1 and 2. Average individual changes in trait values (% of pre-experimental level) are shown in Appendix 3 (see Supplementary material).

to boosting of any other physiological aspect of drug-treated birds.

On the second day of anticoccidial medication (day 16), wild-grown feathers from the breast and tail of the birds were collected for colour measurements. On the morning of day 21 (when the parasite loads of all birds reached zero), the second set of blood samples was taken (time point 'cure' in Figs 1 and 2). A week later

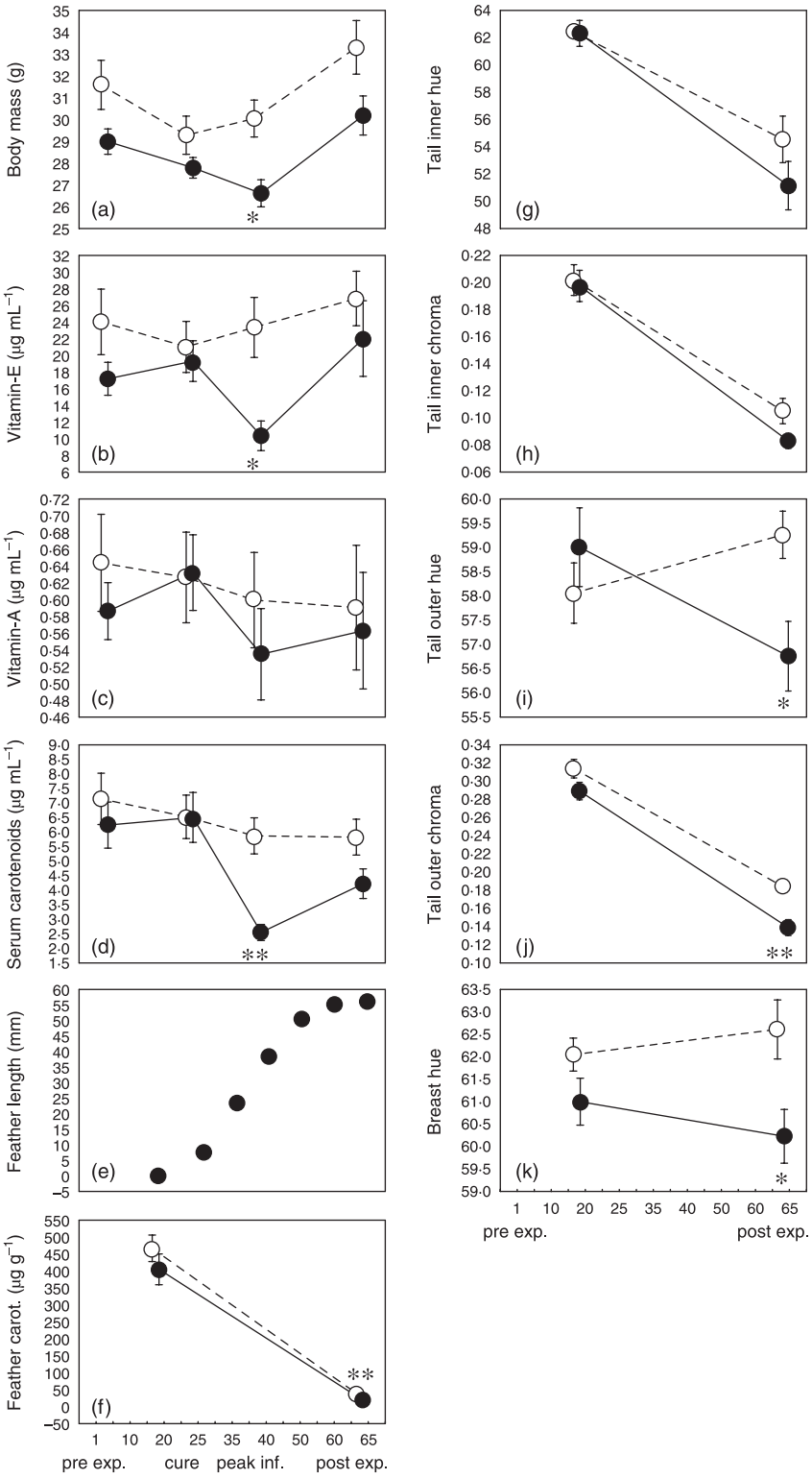

Fig. 2. Effect of manipulation of parasite load upon condition indices and feather coloration of greenfinches. Filled circles, infected group; empty circles, medicated group. Asterisks denote significance of pairwise contrasts between infected and medicated group (*$P < 0{\cdot}05$; **$P < 0{\cdot}001$). The course of experiment in days is noted in *x*-axes of the lower graphs. Sample sizes and statistics are given in Tables 1 and 2. Average individual changes in trait values (% of pre-experimental level) are shown in Appendix 3 (see Supplementary material).

(on day 28), 14 randomly selected birds comprising the infected group were inoculated orally with 2000 sporulated oocysts of *Isospora lacazei* (Labbe). Concurrently, the 14 individuals from the medicated group were subjected to a 5-day medication period (day 28–32) with Vetacox. Hereafter, the birds in the medicated group received further medication with Vetacox in 5-day periods with 2-day intervals between the periods,

until day 52 (medication lasted from days 35–39, 42–46 and 49–53). Eight days after the experimental treatment (drug treatment vs. infection; day 36) the third set ('infection/cure') of blood samples was taken. On day 63 (last time point in Figs 1 and 2), the laboratory-grown feathers had reached their full length and were collected. On the morning of the same day, the last set of blood samples (post-experiment) was also collected.

To assess endoparasite loads, we collected faecal samples during 3 days around the blood samplings (days 1–3, 19–21, 34–36 and 61–63). The individual growth rate of laboratory-grown tail feathers was recorded with the weekly interval, starting from 11 days (day 27) after the removal of the wild-grown tail feathers (Fig. 2e).

We did not use a third control group of totally untreated birds because it appeared impossible to maintain greenfinches in captivity as parasite-free without anticoccidial treatment. A pilot study showed that greenfinches kept in captivity without treatment become as intensively infected (due most probably to relapse of chronic latent infections) as the birds which were experimentally infected with 2000 oocysts in the current experiment. Reference values of 15 mainly haemato-serological condition indices of greenfinches not treated against coccidia have been reported by Hõrak *et al*. (2002 and 2003).

PARASITES

Coccidia of the genus *Isospora* are obligate intracellular parasitic protozoa, following the typical apicomplexan life cycle. A host becomes infected when it ingests oocysts that have been passed in the faeces of another host. The oocyst excysts in the epithelial cells of intestinal mucosa and liberates sporozoites from its contents. The sporozoites penetrate the cells of the host's small intestine and reproduce asexually. In case of passerine birds, the first-generation sporozoites may also invade liver, spleen and lungs (atoxoplasmosis, e.g. Giacomo *et al*. 1997). In the epithelial cells of intestine, each generation of asexual reproduction produces multiple merozoites that infect new cells. This stage of the infection can result in destruction of massive numbers of cells in the host's small intestine and, ultimately, lead to the host's death (e.g. Box 1977; Sironi 1994). Some of the merozoites that enter the host's cells transform into gametocytes. The gametocytes transform into gametes, the gametes fuse, and the resulting zygote begins to develop into an oocyst. The developing oocyst escapes from the host's cell, and it is passed in the host's faeces.

To identify the coccidia infecting greenfinches, 60 oocysts, taken from a mix of oocysts collected from the eight individuals, were examined with phase contrast microscope (magnifications 40×15 and 90×10 with oil immersion). Species was determined as *Isospora lacazei* according to Scholtyseck (1979) and Levine (1982). Isosporan oocysts to be used for oral inoculation were collected from the faecal samples of eight birds that had heavy natural infections during the 5-day period before the experiment. Following collection, faeces were mixed with tap water in a ratio of 2 mL of water per 1 g of faeces. The mixture was held at room temperature for 1 h. Consecutively, the mixture was drained through gauze into separate 10 mL polypropylene tubes. Oocysts were separated from faecal particles by three successive washing procedures with 10 mL of water by centrifugation at 2500 r.p.m. for 7 min. During each washing procedure, the supernatant was removed after centrifugation, leaving 1 mL residue with oocysts. After the third washing the oocysts were floated by dissolving the 1 mL residue into 10 mL of a 45% table sugar (saccharose) solution. This solution was centrifuged at 3000 r.p.m. for 15 min. Then, a 0·5-mL aliquot was collected from the surface of the solution, where the oocysts were floating, and diluted in 10 mL of water. The sugar was removed from this solution by washing the oocysts with water. This procedure gave us clean oocysts, suspended in 1 mL of water. Oocysts were preserved in 20 mL of 2% potassium dichromate ($K_2Cr_2O_7$) solution at room temperature and aerated daily. Sporulation of the oocysts was registered 5 days after collecting by microscopic observation. For the experimental infestation, the oocysts were washed with water to remove potassium dichromate from the solution. The birds of the infected group were inoculated orally with 2000 oocysts diluted in 120 µL of water.

To determine individual parasite loads, individual faecal samples were collected around 1800 h (due to diel periodicity in oocyst shedding; see, e.g. Brown, Ball & Holman 2001). The collected faecal samples were weighed to the nearest 0·01 g with an electronic balance (Mettler Toledo AB-S, Greifensee, Switzerland), suspended in 1 mL of water and held at room temperature for 30 min. Then, the solution was drained through gauze into individual tubes and centrifuged at 1500 r.p.m. for 7 min. The supernatant was removed and 0·2 mL of saturated NaCl water solution was added to the 0·5 mL residue. The number of oocysts was counted using the Goryayev haemocytometer (volume = 0·0018 mL) and their concentration was expressed as number of oocysts per gram of faecal sample. The average of two counts from individual faecal samples was used as the estimate of daily oocyst production rate. The averages of parasite counts of 3 days, preceding the blood-sampling days (days 1, 21, 36 and 63), were used in statistical analyses. This was carried out to account for possible daily variation in individual oocyst counts and also because on day 21 all individual samples appeared parasite-free, which would have not permitted the use of repeated-measures ANOVA for analysis of variation in individual parasite load.

LEUCOCYTE COUNTS

For counting leucocytes, a drop of blood was smeared onto two individually marked microscope slides,

air-dried, fixed in absolute methanol and stained with azure-eosin (Reachim, Chostkinsk, Russian Federation). The proportion of different types of leucocytes was assessed on the basis of examination of 100 leucocytes under 1000 × magnification under oil immersion. Estimates of the total white blood cell count (WBC) were obtained by counting the number of leucocytes per approximately 10 000 erythrocytes. Differential leucocyte counts were obtained by multiplying their proportions with WBC. The repeatabilities of leucocyte counts obtained in this method were found to be reasonably high and significant (Ots, Murumägi & Hõrak 1998).

SEROLOGICAL ANALYSES

Total plasma protein concentration was determined in a photometric colourimetric test using the Biuret method. Standard agarose gel electrophoresis with the REP System (Helena Laboratories, Beaumont, TX, USA) was used for detection of major protein groups (see Ots *et al*. 1998 for details). Due to difficulties in separating the pre-albumin fraction from albumin, summed concentrations of both are reported and termed albumin concentration. Serum triglyceride concentrations were determined by enzymatic colourimetric test (GPO-PAP) using Biosub ® TG kit (Biocon ®, Germany).

Vitamins A, E and carotenoids were extracted from plasma with hexane after protein precipitation with ethanol as described previously by Surai *et al*. (2001). Vitamins E (α-tocopherol) and A (retinol) were determined as described by Surai, Noble & Speake (1996) using a high performnce liquid chromatography (HPLC) system (Shimadzu Liquid Chromatograph, LC-10AD, Japan Spectroscopic Co., Ltd, with JASCO Intelligent Spectrofluorometer 821-FP) fitted with a Spherisorb, type S30DS2, 3μ C18 reverse phase HPLC column, 15 cm × 4·6 mm (Phase Separations Ltd, UK). Chromatography was performed using a mobile phase of methanol/water (97 : 3, v/v) at a flow rate of 1·05 mL min^{-1} with a fluorescence detection of α-tocopherol and retinol. Calibration was performed using α-tocopherol and retinol solutions in methanol. Tocol was used as an internal standard. Total carotenoids were determined from the same extract using the same HPLC system, but fitted with a Spherisorb, type S5NH2 5μ C18 reverse phase HPLC column, 25 cm × 4·6 mm (Phase Separations Ltd, UK). Chromatography was performed using a mobile phase of methanol/water (97 : 3, v/v) at a flow rate of 1·5 mL min^{-1}. Total carotenoids were detected at 445 nm as a single peak using lutein as a standard.

FEATHER COLORATION

For the analysis of plumage colour, two to four breast feathers and one, right outermost (6th) tail feather was collected from each individual. In the absence of a feather on the right side of the tail (in three cases) the feather sample was taken from the left side of the tail. The wild-grown breast feathers were collected from the standard position: the midpoint between the middle-part of the sternum and the edge of the wing. Laboratory-grown feathers were collected from the same positions. Collected feathers were placed into a plastic bag and stored in the dark until measurements were carried out. Locations of colour measurements are indicated in Appendix 1 (see Supplementary material). Colour was measured from the feathers placed on a black background, in an area of approximately 1 mm^2, of the visible surface of the feather, using a spectrophotometer (Ocean Optics S2000) as described by Saks *et al*. (2003). To estimate colour, we calculated values of hue and chroma (see Endler 1990 for details). Hue can be understood as the everyday meaning for colour (yellow, green, red, etc.). Hue is a correlate of the shape of the reflectance spectrum, measured in degrees, around a circular spectrum (colour wheel) whereas chroma is a measure of the 'purity' or 'saturation' of a colour. Details for calculations of colour parameters are given in Saks *et al*. (2003). The repeatabilities (Lessells & Boag 1987) of different colour measurements averaged at 0·74 ± 0·12 (SD) and ranged from 0·50 to 0·88 (Appendix 2; see Supplementary material). In analyses, we use values of hue and chroma for tail feathers and only hue for breast feathers because the chroma of the one set of breast feathers (wild-grown, r between two different pairs of feathers) was too poorly repeatable (r = 0·35). As shown previously in the same set of feather samples, hue and chroma appeared good indicators of feather carotenoid content. Variation in the average carotenoid concentration of the yellow area of tail feathers explained 32–51% of the variation of in these colour parameters (Saks *et al*. 2003).

FEATHER CAROTENOID CONTENT

The yellow pigmented portions of the sampled feathers were trimmed off and weighed to the nearest 0·00001 g with an electronic balance (Mettler Toledo AG245, Greifensee, Switzerland). The carotenoids were extracted from the feather samples using acidified pyridine (Hudon & Brush 1992), purified, and identified with HPLC as described in Saks *et al*. (2003). The total carotenoid concentration (μg g^{-1}) in each sample was determined by spectrophotometry (Bausch and Lomb Spectronic 1001).

STATISTICS

The effect of experimental manipulation of coccidian infection in greenfinches was examined with repeated-measures ANOVA, assuming that the effect of treatment will be revealed by a significant 'time × treatment' interaction term. Additionally, we examined the differences between infected and medicated birds by calculation of pairwise contrasts between group averages during peak infection phase and post-experimental phase. Significant differences are indicated by asterisks in Figs 1 and 2; detailed statistics are presented in Table 2. In the case

of three variables (lymphocyte count, body mass, vitamin A concentration) the assumption of sphericity of variance–covariance matrix was violated. In these cases, the significance values were based on Geisser–Greenhouse correction. To obtain normal distribution of variables or homogeneity of variances (according to Levene's test), values for parasite count, WBC, heterophile count and serum globulin concentration were log-transformed. However, data for all variables (except for parasite counts) are presented as untransformed in Figs 1 and 2 in order to provide reference data. Data are presented as mean ± SD; all *P*-values are for two-tailed tests. Sample sizes vary between analyses due to our inability to obtain all required parameters from all birds throughout the study. This holds especially for fat-soluble antioxidants where the sample size (eight medicated and eight infected birds) is considerably smaller than that for the rest of variables.

Results

In the pre-experimental period, 89% (25/28) of all individuals were infected with coccidia. The average intensity of infection was 3595 ± 8231 oocysts g^{-1}. After 5 days' treatment with a coccidiostat, the average parasite loads in both experimental and control groups dropped to pre-experimental levels (Fig. 1a), with all faecal samples appearing parasite-free on day 6 after the start of medication. Eight days after infecting the experimental group (and continued drug treatment in the control group), the average difference in oocyst output between two bird categories was 263 times (infected 87812 ± 123347; medicated 334 ± 674). Oocyst output of infected group in the peak infection phase rose 16 times ($P = 0{\cdot}010$; $Z = 2{\cdot}55$; Wilcoxon's matched pairs test) above the pre-experimental level (of 1691 ± 1920 oocysts g^{-1}). Parasite load in the infected group remained high for 35 days following experimental infection (fourth time-point in Fig. 1a), while in the control group the parasite load started to increase slowly after withdrawal of medication. On the 10th day after the last medication, 50% of the medicated birds were already shedding oocysts. Group average oocyst output by that time was 4129 ± 9108, which approached pre-experimental levels (5363 ± 11185). Hence, our experiment resulted in successful manipulation of coccidian parasitism in greenfinches, the differences in infection intensity between experimental categories being highest in the third time point of sampling (Table 1, Fig. 1a). In that period (termed hereafter as peak-infection phase), the experimental group had been infected for 8 days while the control group was continuously receiving medication.

Based on significant 'time × treatment' interaction terms in Table 1, the following physiological parameters were affected by the experimental manipulation of coccidian parasite load. The peak-infection phase was followed by a transient increase in total leucocyte count (Fig. 1b), which was due mainly to heterophils (infected birds had a 36% higher concentration of circulating heterophils than medicated birds; Fig. 1c). The increase in lymphocyte count in our experimental group (Fig. 1d) was not significant (Table 1), nor did the treatment significantly affect heterophile/lymphocyte ratio (Fig. 1e). Total serum protein (Fig. 1f) and globulin concentration were not significantly affected by the treatment (although a tendency for increased globulin levels in the infected group is suggested by inspection of Fig. 1g). Serum albumin content was 24% lower among infected birds than controls in the peak infection phase (Fig. 1h). This drop in serum albumin evidently led to a significantly lower albumin/globulin ratio in the infected group in the peak infection phase (Fig. 1i). Similarly to that of albumin, serum triglyceride concentration in the infected group also decreased during peak infection, being 34% lower than pre-infection level and 46% lower than in the control group (Fig. 1j).

Average body mass in infected group was consistently lower than in the medicated group (Fig. 2a); however, pairwise contrasts indicated a significant difference only in the peak infection phase ($F_{1,25} = 10{\cdot}4$, $P = 0{\cdot}004$). Thus, by that time infected birds had lost on average of 2·4 ± 3·0 g (i.e. 8%) of their pre-experimental mass, while the medicated birds had lost on average 1·5 ± 1·8 g (i.e. 5%), suggesting that experimental infection had also an effect on body mass in greenfinches. During the peak infection phase, infected birds had 40% lower serum vitamin E concentrations than in the pre-experimental period and 56% lower vitamin E levels than in the medicated group (Fig. 2b). Vitamin A concentrations were not obviously affected by the experimental procedure (Fig. 2c). Serum carotenoid concentration (Fig. 2d) in the infected group dropped abruptly during the peak infection (59% compared to initial levels and 57% compared to medicated group).

Feather moult (Fig. 2e) occurred at a time when the medicated group received continuous medication and the infected group remained heavily infected (Fig. 1a). Compared to wild-grown feathers, the average carotenoid content of laboratory-grown feathers was 93% lower than initial in both groups of birds. Nevertheless, the total carotenoid content by the end of experiment was significantly (52%) lower in the infected compared to the medicated group (19·1 ± 10·9 μg g^{-1} vs. 39·8 ± 15·6 μg g^{-1}; Fig. 2f).

We found no differences between the treatment groups with respect to the colour of the inner vane of tail feathers (Fig. 2g,h). The coloration of the outer vane was obviously more affected by the treatment because its hue decreased by 4% (as compared to wild-grown feathers) in the infected group, while in the medicated group the value of hue increased by 2% compared to the initial (Fig. 2i). As a result, laboratory-grown feathers of infected birds had 4% lower values of hue than those of medicated birds. A significant effect of manipulation was also manifested by a significant 'time × treatment' interaction term in Table 1. The chroma of the outer vane was consistently higher (i.e. among both wild- and laboratory-grown feathers) in the group of medicated

Table 1. Effect of manipulation of parasite load upon condition indices and plumage coloration of greenfinches. Direction of the effects is presented in Figs 1 and 2. Med. and Inf. stand for sample sizes of medicated and infected group, respectively

Trait	Type of response	*F*	*P*	*n*
Parasite load	treatment	11·95	0·002	Med. 13
	time	25·38	< 0·00001	Inf. 14
	time × treatment	10·03	0·00001	
WBC	treatment	0·52	0·482	Med. 8
	time	38·66	< 0·00001	Inf. 8
	time × treatment	4·30	0·0099	
Heterophil count	treatment	1·78	0·203	Med. 8
	time	14·37	< 0·00001	Inf. 8
	time × treatment	4·02	0·013	
Lymphocyte count	treatment	0·70	0·418	Med. 8
	time	14·38	0·0009	Inf. 8
	time × treatment	2·61	0·121	
Heterophil/lymphocyte ratio	treatment	0·78	0·393	Med. 8
	time	0·23	0·873	Inf. 8
	time × treatment	0·66	0·582	
Total protein concentration	treatment	2·12	0·158	Med. 14
	time	2·26	0·089	Inf. 13
	time × treatment	1·03	0·383	
Albumin concentration	treatment	3·60	0·070	Med. 14
	time	3·92	0·012	Inf. 12
	time × treatment	3·75	0·015	
Globulin concentration	treatment	0·42	0·523	Med. 14
	time	2·91	0·040	Inf. 12
	time × treatment	1·69	0·178	
Albumin/globulin ratio	treatment	3·55	0·072	Med. 14
	time	7·55	0·0002	Inf. 12
	time × treatment	7·997	0·0001	
Triglyceride concentration	treatment	6·16	0·020	Med. 13
	time	38·61	< 0·00001	Inf. 13
	time × treatment	17·65	< 0·00001	
Body mass	treatment	5·73	0·025	Med. 14
	time	24·18	< 0·00001	Inf. 13
	time × treatment	1·69	0·195	
Vitamin E concentration	treatment	3·18	0·096	Med. 8
	time	4·18	0·011	Inf. 8
	time × treatment	2·48	0·074	
Vitamin A concentration	treatment	0·26	0·615	Med. 8
	time	1·69	0·203	Inf. 8
	time × treatment	0·49	0·622	
Plasma carotenoid concentration	treatment	2·84	0·114	Med. 8
	time	21·73	< 0·00001	Inf. 8
	time × treatment	7·44	0·0004	
Feather carotenoid concentration	treatment	1·88	0·183	Med. 13
	time	183·84	< 0·00001	Inf. 12
	time × treatment	0·48	0·497	
Tail inner hue	treatment	2·03	0·168	Med. 13
	time	44·56	< 0·00001	Inf. 11
	time × treatment	1·32	0·263	
Tail inner chroma	treatment	1·87	0·186	Med. 13
	time	107·22	< 0·00001	Inf. 11
	time × treatment	0·75	0·396	
Tail outer hue	treatment	1·45	0·241	Med. 13
	time	0·60	0·448	Inf. 12
	time × treatment	6·49	0·018	
Tail outer chroma	treatment	23·25	0·00007	Med. 13
	time	194·61	< 0·00001	Inf. 12
	time × treatment	1·03	0·320	
Hue of breast feathers	treatment	6·63	0·017	Med. 14
	time	0·07	0·792	Inf. 12
	time × treatment	2·77	0·109	

Table 2. Pairwise contrasts (from ANOVA) of condition indices between infected and medicated birds during peak infection phase and of colour measurements after the experiment. Significant differences between averages of drug-treated and infected birds are also indicated by asterisks in Figs 1 and 2

Trait	d.f.	*F*	*P*
Parasite load	1; 25	21·92	0·00009
WBC	1; 14	4·29	0·057
Heterophil count	1; 14	7·10	0·019
Lymphocyte count	1; 14	2·16	0·164
Heterophil/lymphocyte ratio	1; 14	1·41	0·256
Total protein concentration	1; 25	3·78	0·063
Albumin concentration	1; 24	13·48	0·001
Globulin concentration	1; 24	3·62	0·069
Albumin/globulin ratio	1; 24	34·98	< 0·00001
Triglyceride concentration	1; 24	52·23	< 0·00001
Body mass	1; 25	10·42	0·004
Vitamin-E concentration	1; 14	10·48	0·006
Vitamin-A concentration	1; 14	0·67	0·425
Plasma carotenoid conc.	1; 14	23·99	0·0002
Feather carotenoid conc.	1; 23	14·44	0·0009
Tail inner hue	1; 22	1·88	0·184
Tail inner chroma	1; 22	3·73	0·067
Tail outer hue	1; 23	8·59	0·008
Tail outer chroma	1; 23	19·48	0·0002
Hue of breast feathers	1; 24	6·95	0·014

birds (Fig. 2j). However, inspection of pairwise contrasts revealed that wild-grown feathers did not differ significantly between the groups ($F_{1;23} = 3·0$; $P = 0·095$), while among laboratory-grown feathers, infected birds had significantly lower values of chroma than medicated birds ($F_{1;23} = 19·5$; $P = 0·0002$; 22% difference). Similar to the results for outer-vane chroma, the hue of the breast feathers was, in both cases, higher in the group of medicated birds (Fig. 2k). Again, pairwise contrasts indicated that wild-grown feathers did not differ significantly between the groups ($F_{1;24} = 2·8$; $P = 0·105$), while among laboratory-grown feathers, infected birds had significantly lower values of hue than medicated birds ($F_{1;24} = 7·0$; $P = 0·014$; 4% difference).

Discussion

EFFECT OF INFECTION ON HOST PHYSIOLOGY

Our experimental medication and infection protocol with coccidian parasites resulted in successful manipulation of parasite oocyst output in captive greenfinches. Prevalence of isosporan oocysts dropped from 89% of pre-experimental level to 0% 6 days after the start of medication. It should be noted, however, that this medication did not result in complete clearance of parasites, as by the 10th day after withdrawal of medication, 50% of the medicated birds had again started to shed parasitic oocysts. This is not surprising, because the action of sulphonamide drugs is coccidiostatic, i.e. based upon the prevention of the multiplication of microbes. However, the effects of medication were detectable for at least 10 days after withdrawal because in the final sampling point of the experiment (35 days after infection of the experimental group and 10 days after withdrawal of medication in the medicated group), significant differences in average oocyst loads of two bird categories were still present (Fig. 1a). Most importantly, the treatment with Vetacox was sufficient to cause more than a 200-fold difference in oocyst output between medicated and infected groups during the peak infection phase. Thus we believe that the differences in physiology and appearance between our experimental groups emerged due to suppression of coccidian multiplication in drug-treated birds. This is also indicated by the clinical profile of infected birds, which is typical of intestinal infection.

Among the most conspicuous symptoms accompanying the peak phase of infection were the sudden and transient decreases of serum albumin, triglyceride, vitamin E and carotenoid concentrations (Figs 1h,j and 2b,d). The most probable explanation for these parallel patterns is the reduction of digestive and absorptive capacity of mucosa, which follows the destruction of epithelial cells during the reproduction of the parasite (e.g. Ruff & Fuller 1975; Hoste 2001). Isosporan parasites of cardueline finches have been shown to damage most extensively the duodenal and jejunal part of the intestine (Giacomo *et al.* 1997). In domestic chicken (Turk 1974), these intestinal compartments have been shown to be responsible for absorption of proteins (jejunum) and fats (duodenum), as well as vitamin E and carotenoids (Surai 2002), which would explain the drop in serum albumin, triglycerides and fat-soluble antioxidants (carotenoids and vitamin E). In humans and domestic chickens, lipid malabsorption causes decreases in plasma carotenoids and is a common cause of decreased plasma vitamin E, leading to deficiency syndromes (reviewed in Allen & Fetterer 2002a). A recent study on chickens infected with *Eimeria maxima* showed that the reductions in plasma vitamin E (and carotenoid) levels during acute phase of infection depended on the dose of inoculation and that providing high dietary levels of vitamin E did not afford any antioxidant protection to hosts (Allen & Fetterer 2002a). With regard to serum albumin, the reduction of its concentration during the peak infection phase could be caused additionally by leakage of this protein into the intestinal lumen because of the damaged mucosa (Allen & Fetterer 2002a). Altogether, these infection-induced effects upon host nutritional status were evidently severe enough to lead to significantly lower body mass in the infected compared to the medicated group during the peak infection phase (Fig. 2a). Furthermore, an additional reason for the drop of serum carotenoids and vitamin E during the peak infection phase could be due to their usage in antioxidant defence against free radicals produced during the immune response (e.g. Allen 1997). For instance, coccidians may stimulate phagocytic cells of the host's immune system to produce superoxide anion that by itself, or as an initiator of free radical cascades, causes pathology and oxidizes carotenoids

(Allen, Danforth & Levander 1997) and/or depletes vitamin E storages (Allen & Fetterer 2002a). Yet another reason for the reduction in blood carotenoid levels due to infection may relate to depressed synthesis of high density lipoproteins (the main carriers of carotenoids and vitamin E) in the intestinal mucosa, as has been shown during *E. acervulina* infections in chickens (Allen 1987). These pathways, however, would not explain the reductions in body mass or serum albumin and triglyceride levels during the peak infection phase. Notably, with respect to the effect of coccidian infection upon the body mass of birds, our results resemble two other studies of cardueline finches (greenfinches, Cooper *et al*. 1989 and American goldfinches, McGraw & Hill 2000). In the latter study, infected birds also reduced food consumption, suggesting that infection-induced anorexia may play an additional role in the loss of body mass due to coccidiosis (see also Allen 1987).

With regard to the effect of infection upon leucocyte counts, our results differ from those of Rose, Hesketh & Ogilvie (1979) where primary eimerian infections in chickens and rats resulted in a biphasic increase in leucocyte (both heterophile and lymphocyte) count with intermittent lymphopenia during the peak infection phase. In greenfinches, however, only a marked heterophilia could be detected (Fig. 1c). A possible reason for this difference is that animals in the study of Rose and colleagues were immunologically naive to eimerian parasites, while 89% of greenfinches naturally hosted chronic isosporan infections. Our birds were reinfected later with a mixture of parasitic strains from different hosts, which is expected to induce different immune responses than occurred during primary infections. The increase of peripheral heterophil count in greenfinches may be associated with increased traffic of these phagocytozing cells to the intestinal mucosa, where they participate in the removal of debris (e.g. destroyed host tissues, dead parasites; e.g. Rose *et al*. 1979). Such phagocytotic processes are often linked to remarkable tissue damage caused by the production of proteolytic enzymes and free radicals (e.g. Klasing & Leshchinsky 1999). Notably, the pathogenicity of isosporan parasites in black siskins (*Carduelis atrata*) has been associated with abnormal and inadequate cell-mediated responses to parasites (Giacomo *et al*. 1997).

To sum up, the changes in host physiology, similar to those observed in this study, have been recorded previously in domestic chicken infected with various species of *Eimeria* (e.g. Ruff & Fuller 1975; Conway *et al*. 1993; Allen & Fetterer 2002a). Our study provides the first evidence that isosporans of wild birds (whose biology differs from that of *Eimeria* species) can cause similar physiological effects as eimerian parasites in poultry. Furthermore, this study outlines the specific diagnostic values of serum albumin, triglycerides, carotenoids and vitamin E as a markers of the severity of isosporan infection. These markers would be useful in future studies examining the pathogenicity of isosporan parasites in experimental infection protocols.

EFFECT OF INFECTION ON FEATHER COLOUR AND CAROTENOID CONTENT

The laboratory-grown tail feathers of infected birds contained 52% less carotenoids than those of medicated birds, and their outer vanes had 22% smaller values of chroma and 4% smaller values of hue than medicated birds (Fig. 2f,i,j). Similarly, the laboratory-grown breast feathers of infected birds had 4% smaller values of hue than medicated birds (Fig. 2k). Therefore, we can conclude that experimental coccidian infection indeed affected feather carotenoid content and colour in captive greenfinches. Hence, our results present direct experimental support for one of the basic premises of parasite-mediated sexual selection, namely that parasites affect the expression of secondary sexual traits. Along with the studies of two other cardueline finches (Brawner *et al*. 2000; McGraw & Hill 2000), this is another piece of evidence illustrating the modification of carotenoid-based plumage colour by coccidian parasites. More generally, our results are in line with those of experimental infections of other taxa. For instance, experimentally parasitized male sticklebacks (*Gasterosteus aculeatus*; Milinski & Bakker 1990) and guppies (*Poecilia reticulata*; Houde & Torio 1992) developed reduced carotenoid-based breeding coloration. Similarly, male laboratory mice (*Mus musculus*) infected with coccidian parasite *E. vermiformis* lost the attractiveness of the odour of their urine for the females (Kavaliers & Colwell 1995).

DECLINE IN COLOUR AND CAROTENOIDS INDEPENDENTLY OF INFECTION

Besides the effect of experimental infection it should be noted, however, that experimentally induced differences in both feather carotenoid content and colour were relatively small compared to differences between wild-grown and laboratory-grown feathers. On average, laboratory-grown tail feathers of both infected and medicated birds contained 93% less carotenoids than wild-grown feathers (Fig. 2f), while the chroma of outer and inner vanes of tail feathers decreased, respectively, 47% and 50% (Fig. 2j,h) and hue of the inner vane decreased by 15% (Fig. 2g) in captivity. Hence, some factor related to captivity (or season) *per se* affected feather carotenoid content and some components of colour much more strongly than coccidian infection. Which factors could account for this effect? We can see two most likely (not mutually exclusive) explanations here.

First, the pure sunflower seed diet used in this study might have been an insufficient source of dietary carotenoids required for the development of bright yellow plumage. The total concentration of carotenoids in sunflower seeds is about 1 $\mu g\ g^{-1}$; the main carotenoid is lutein (65% of total), followed by 34% of zeaxanthin and 2% of β-carotene (McGraw *et al*. 2001). This is generally thought to be low for most edible plant parts

(Goodwin 1980); in fact, other common bird seeds have 4–5 times more carotenoids than this (e.g. white millet; McGraw *et al*. 2001). Fed a low concentration of dietary carotenoids, captive greenfinches in this study accumulated low levels of serum carotenoids (6–7 μg mL^{-1}; Fig. 2d), which probably contributed to their faded plumage colour. A study of a closely related species, the American goldfinch (*C. tristis*), showed that wild-caught goldfinches circulate nearly two to three times the concentration of carotenoids in blood (15–30 μg mL^{-1}) than do captive birds fed a sunflower seed diet during moult and protected from coccidian infections (6–9 μg mL^{-1}; Gregory 2002). We cannot test this idea adequately in greenfinches, however, because we are currently not aware of the natural carotenoid concentration in the blood of greenfinches during moult.

Secondly, even at the level of sufficient carotenoid content, the diet of sunflower seeds might have been poor in some other essential micronutrients required for biotransformation, transportation and deposition of carotenoids in the feathers. For instance, the levels of β-carotene and vitamin A are very low in sunflower seeds (Harper & Skinner 1998; McGraw *et al*. 2001), while the levels of riboflavin (vitamin B_2) are considered to be relatively low in seeds (Harper & Skinner 1998). The passage of carotenoids from food to feathers involves multiple steps and mediators, such as substrate-specific membrane proteins in the gut, carrier proteins for transportation and enzymes for bioconversion. As pointed out by Hill (2000), the details of all these processes remain virtually unstudied but clearly there exists the potential for nutritional stress, independent of carotenoid availability to affect plumage coloration of birds. The best example for this is the study of Hill (2000) where food-restricted male house finches (*Carpodacus mexicanus*) grew less red plumage than *ad libitum*-fed controls, despite receiving equal carotenoid doses in their diet.

Ultimately, it is important to recognize that our study reflects the relationship between health and appearance of greenfinches under captive conditions, where carotenoid access and status was much lower than in the wild. There is evidence in a congeneric finch, the American goldfinch, that carotenoid-based coloration in the wild is linked correlationally to coccidial burdens as it was in our study (Olson 1996), but whether experimental coccidian infection would have pronounced effects upon ornamental traits in greenfinches maintained on more natural diets rich in carotenoids and micronutrients remains to be answered in future studies.

LACK OF PROTECTIVE IMMUNITY

Yet another interesting aspect of this study was that drastic effects of experimental infection upon physiology of greenfinches occurred despite the fact that most (89%) of individuals were already infected with isosporan parasites before the experimental treatment. Thus chronic isosporan infection does not appear to provide sufficient protective immunity against subsequent infections with the same parasite. This result may seem surprising, given that avian coccidia are considered highly immunogenic and primarily infections can stimulate solid immunity to homologous challenges (reviewed in Martin *et al*. 1997; Allen & Fetterer 2002b). However, this strong impact of experimental infection in chronically infected greenfinches can be easily reconciled with the concept of antigenic diversity of different strains of the parasite, which means that infection of hosts within one subpopulation of a pathogen fails to induce cross-protective immunity against another. Immunologically relevant genetic diversity within a pathogen population allows different strains of a pathogen to infect hosts that are immune to other antigenic variants and is driven most probably by the acquired immune response of the host population. Under this scenario, host genotype and infection history act in concert to determine the level of cross-protective immunity generated by infection (reviewed by Apanius *et al*. 1997; Smith *et al*. 2002).

Nothing is known about strain specificity of avian *Isospora*, although similarly to those of this study, the results of Brawner *et al*. (2000) and McGraw & Hill (2000) suggest that health impact of experimental infection can be induced in already infected individuals. Notably, recent studies with *E. maxima* in chickens (Smith *et al*. 2002) have demonstrated full protective immunity against the reinfection with the same strain of the parasite, while cross-protection against the heterologous parasite strain varied from zero to almost 100%, depending on host genetics. This is actually the situation predicted by the original Hamilton–Zuk hypothesis of host–parasite arms races, namely that the simultaneous occurrence of genetic polymorphism among both hosts and parasites determines infection success. In fact, coccidia seem to appear ideal parasites for testing the Hamilton–Zuk hypothesis because hosts are likely to acquire continuously multiple infections from diverse strains. Simultaneous coexistence of these different strains within the digestive system of hosts will further expand the antigenic diversity of parasites due to recombination in the process of sexual reproduction within the host. Furthermore, the chronic nature of coccidian infection (which still does not exclude outbreaks of heavy mortality) stresses further the suitability of these parasites as potential causative agents of parasite-mediated sexual selection, because such infections allow males to display and females to benefit from parasite-resistant genes (Hamilton & Zuk 1982). Consistent with this prediction, females have been shown to base their mate choice on ornamental traits affected by coccidian infection in ring-necked pheasants (*Phasianus colchicus*, Hillgarth 1990), wild turkeys (*Meleagris gallopavo*, Buchholz 1995), American goldfinches (Johnson, Dalton & Burley 1993) and house finches (reviewed by Hill, Inouye & Montgomerie 2002).

In conclusion, we have demonstrated drastic effects of infection with *I. lacazei* upon the physiology and expression of carotenoid-based plumage coloration in

greenfinches. We specified that reduced absorption of nutrients (including carotenoids and vitamin E) appeared the plausible main mechanism responsible for these effects. Furthermore, we identified several sensitive haemato-serological markers that can be used for diagnostics of the physiological impact of infection. Our results indicate that the isosporan parasites appear an excellent model system for elucidating the physiological and genetic mechanisms of parasite-mediated sexual selection. However, this study also showed that the pathological effects of coccidian infection do not appear necessarily the single (or even the major cause) for fading of carotenoid-based plumage coloration, which calls for further experiments in this area.

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Supplementary material

The following material is available from: http://www.blackwellpublishing.com/products/journals/suppmat/JAE/JAE870/JAE870sm.htm

Appendix 1. Tail feathers of male greenfinches: their carotenoid content (mg g^{-1}) and colour measurements.

Appendix 2. The repeatabilities of the feather colour measurements.

Appendix 3. Effect of manipulation of parasite load upon condition indices and feather coloration of greenfinches, calculated as mean individual changes (%) compared to pre-experimental state (100%).

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