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Carotenoids modulate the effect of coccidian infection on the condition and immune response in moulting house sparrows

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SUMMARY

In the present study, we experimentally manipulated coccidian parasitism and dietary carotenoid availability in a fully factorial experiment in male house sparrows (*Passer domesticus* Linnaeus), and tested whether carotenoid supplementation reduces the cost of parasitism in terms of condition, moult and immune responses. We found that coccidians have a significant but transient negative effect on body mass, which can be reduced if birds have access to carotenoid supplementation in their diet. Experimental manipulation had no significant effect on the moulting parameters of the birds measured following coccidian infestation and during the whole moulting period. Carotenoid supplementation increased the plasma carotenoid concentration in both infested and medicated birds treated with a coccidiostatic drug; however, after two months exposure to parasites, plasma carotenoid concentration increased only in the carotenoid-supplemented and medicated group whereas no difference was observed between the carotenoid-supplemented and infested and non-supplemented groups. On the contrary, coccidian infestation was not affected by carotenoid supplementation. Experimental infestation decreased the antibody response to sheep red blood cells (SRBCs), although no significant effect was observed in the capacity of the birds to respond to a mitogenic challenge with phytohemagglutinin. Within the experimentally infested groups birds with carotenoid-supplemented food tended to have an increased anti-SRBC humoral immune response. The positive correlation between coccidian infestation and the strength of the humoral immune response against SRBCs in the non-supplemented and infested groups indicates that this part of the immune system plays an important role in defence against these parasites.

Key words: aviary, carotenoid supplementation, experimental infection, humoral immune response, *Isospora*, *Passer domesticus*.

INTRODUCTION

Moult is recognised as one of the most demanding activities of birds (Lindström et al., 1993; Klaassen, 1995), which is costly due to predation, energy and nutrient expenditure (Lindström et al., 1993; Jenni and Winkler, 1994; Klaassen, 1995; Pap et al., 2008). Therefore, parasite infections, which affect the condition of the birds, may seriously reduce the fitness through an interaction with the process of moult (Langston and Hillgarth, 1995). Sedentary birds perform their annual complete moult following reproduction, when due to the costly parental activity the immune system is suppressed and consequently the risk of infection is increased (Hasselquist, 2007). We expect infestation to reduce the resources available for moult and hence to have an almost year-round effect on the fitness, because moult can link current with future performance (Dawson et al., 2000). However, this long lasting negative effect can be mitigated by the carotenoids because of their potential immunoenhancement effect (see below). Possible candidates with negative effects on moult are those groups of parasites that detract resources directly from the host and at the same time stimulate the host's immune system, such as intestinal endoparasites or blood-sucking ectoparasites. Coccidians are intestinal parasites, which can seriously affect the productivity of poultries and the fitness of the wild birds (Allen, 1997; Allen and Fetterer, 2002; Gill and Paperna, 2008; Greiner, 2008), inhibiting the uptake of essential dietary components from the alimentary tube and stimulating the immune system of the host (Hõrak et al., 2004; Baeta et al., 2008; Hill et al., 2009; Mougeot et al., 2009). While infections by intestinal coccidians affect a number of songbird species (e.g. Greiner, 2008), our knowledge about the physiological effects of this parasite, the mechanism of defence and their consequences on the fitness of wild birds is very limited (e.g. Hill and Brawner, 1998; Hõrak et al., 2004; Baeta et al., 2008; Mougeot et al., 2009).

Carotenoids are important biomolecules known to play a major role in the defence against parasites in vertebrates. Their essential role in immune function is attributed to their immunostimulating and immunoregulating effects (Blount et al., 2003; McGraw and Ardia, 2003; Saino et al., 2003; Grether et al., 2004; Peters et al., 2004; Cucco et al., 2006; Kolluru et al., 2006; McGraw et al., 2006; Aguilera and Amat, 2007). The concentration and the availability of carotenoids in the peripheral blood are often negatively related

to parasite infestation (e.g. Hõrak et al., 2004; Martínez-Padilla et al., 2007; Mougeot et al., 2007; Mougeot et al., 2009; Baeta et al., 2008), because parasites may inhibit the uptake of carotenoids from the alimentary canal. As a consequence, parasites may reduce indirectly, through the availability of carotenoids, the immune function of the host. The amount of carotenoids available for the physiological function in immune response has been suggested to be limited (Hill and Montgomerie, 1994; von Schantz et al., 1999) (reviewed by Møller et al., 2000), because carotenoids are only procured from food (Slagsvold and Lifjeld, 1985; Hill and Montgomerie, 1994). Therefore, we should expect that the higher availability of carotenoids can alleviate the negative effect of parasites on fitness through enhancing immune function and hereupon increasing the effectiveness of immunological defence against invading pathogens.

Carotenoids have received particular attention in studies concerning the mechanism of plumage colouration and the evolution of costly traits under the pressures imposed by parasites (e.g. von Schantz et al., 1999; Hill et al., 2004; Hill and McGraw, 2006a; Hill and McGraw, 2006b; Hill et al., 2009); however, the role of these biochemicals in physiological performance, defence against parasites or any measure of fitness in hosts with no carotenoid-based signal have remained less studied (McGraw et al., 2006). Because carotenoids may have important immunostimulating and immunoregulating properties (Møller et al., 2000), animals with no carotenoid-based signal in their colouration can allocate the available carotenoids exclusively toward self-maintenance, growth and reproduction (von Schantz et al., 1999; Blount et al., 2001; McGraw and Ardia, 2003; Catoni et al., 2008). However, the role of carotenoids in regulating many other physiologically costly activities largely ignored in ecological immunology studies remains to be demonstrated.

Because of our limited information about the mechanisms of the interaction between coccidian infestation and moult mediated by carotenoid-dependent immune function, in the present study we investigate through a full factorial experiment (1) the effect of coccidian infestation and carotenoid manipulation on condition and moult in the house sparrow (*Passer domesticus* Linnaeus), a common urbanised sedentary bird with no carotenoid colouration, (2) the effect of coccidian infestation on the availability of carotenoids in the peripheral blood, (3) the possible relationship between two different components of the immune system and coccidian infestation in order to determine the role of immune function in defence against these parasites, and finally (4) the possible effect of carotenoids in enhancing the immune responses and modulating the relationship between immune function and coccidian infestation. Accordingly, we predict that coccidians will reduce the concentration of plasma carotenoids, and will negatively influence the body condition, moult and immune responses; however, if extra carotenoids are supplemented, these effects will be ameliorated (significant coccidian × carotenoid treatment interaction).

MATERIALS AND METHODS

General procedure

Fifty-one adult male house sparrows were caught in two farms near Cluj Napoca (Romania, 46°46′N, 23°33′E) during two capture sessions on the 24 and 26 July 2008 and transported in four outdoor aviaries [5×2×2.5 m (length×width×height)] situated in the campus of the Babes-Bolyai University in Cluj Napoca. Throughout the experiment birds were fed *ad libitum* with a mixture of seeds containing grinded corn, sunflower, wheat and oat. Their food was supplemented with two grated, boiled egg albumens on every even day and with two mealworms per bird on every odd day throughout the experiment. Fresh tap water was provided daily throughout their time in captivity. Before the experiment started none of the birds was moulting. Of the 51 captured individuals two died on 1 September and 17 October 2008 due to unknown reasons. House sparrows were released in good health condition after the experiment had finished.

Experimental protocol

After introduction, birds were allowed to acclimatise for 2–4 days in the aviaries. On the day following the acclimation (day 0=28 July) (Fig. 1), house sparrows were moved to individual outdoor cages in order to quantify the natural infestation level of coccidians. For this, we measured the rate of oocyst-shedding (i.e. number of oocysts per gram of faeces per sample day; see below) during three (0–2) days for individual birds. Birds were allowed to keep visual and acoustic contact held in individual cages in order to maintain interactions between individuals in this highly social species. The next day (day 3) we randomly re-introduced the birds in the four aviaries where they were assigned to one of the four experimental groups as follows: (1) carotenoid non-supplemented × coccidia-infected (Carot–Cocc+; N=13), (2) carotenoid-supplemented × coccidia-infected (Carot+Cocc+; N=13), (3) carotenoid non-supplemented × coccidia-medicated (Carot–Cocc–; N=13), and (4) carotenoid-supplemented × coccidian-medicated (Carot+Cocc–; N=12). Carotenoid treatment started on this day, while the coccidia treatment was implemented on day 40 to test the effect of carotenoid supplementation on the natural infestation of the birds (see below). Carotenoid-supplemented groups received 40 μg g^{-1} carotenoids supplied daily in their food [Oro Glo dry, 11 mg ml^{-1}, lutein and zeaxanthin (20:1, v/v); Kemin France SRL], which is well within the natural range of dietary carotenoid concentration for wild granivorous songbirds (Hill and McGraw, 2006a; McGraw et al., 2006). To our knowledge, no data are available quantifying carotenoid content in the diet of house sparrow, and that is why we calibrated the level of supplementation to that for granivorous songbirds. Food was coated in sunflower oil to ensure that carotenoid powder adhered to and spread homogenously throughout the seeds. The same amount of oil without carotenoids was added to the diet of non-supplemented birds as well. On day 24 we collected 75 μl of blood from the brachial vein in heparinised capillaries to determine the carotenoid concentration in the peripheral blood. The plasma was separated by centrifugation of capillary tubes for 5 min at *ca.* 6200 ***g***, then the plasma was stored at –20°C until analysis. Starting with day 24, we measured again the rate of oocyst-shedding of individual birds for three (24–26) days. Four days later (day 30), all birds were treated with an anticoccidial parasiticide provided in their drinking water for three (30–32) days (Baycox®, Bayer Healthcare, Germany, 2.5 g toltrazuril in 100 ml^{-1} water), and eight days later (day 40) birds in the Cocc+ groups were inoculated with isosporan oocysts, while the other half served as the control, receiving physiological solution only (Cocc–). Because the effect of anticoccidial parasiticide lasts for only a few days (Hõrak et al., 2004) (and P.L.P., C.I.V. and G.Á.C., unpublished), and after which time oocyst-shedding reappear in the faeces due to natural reinfection, we medicated the birds from Cocc– experimental groups against coccidians during every week for two days until the end of the experiment in order to keep the parasites under control (see Fig. 1). Seven days after infestation (day 47) we collected the same quantity of blood from all birds for carotenoids analyses, after that they were injected intramusculary with 100 μl 20% fresh sheep

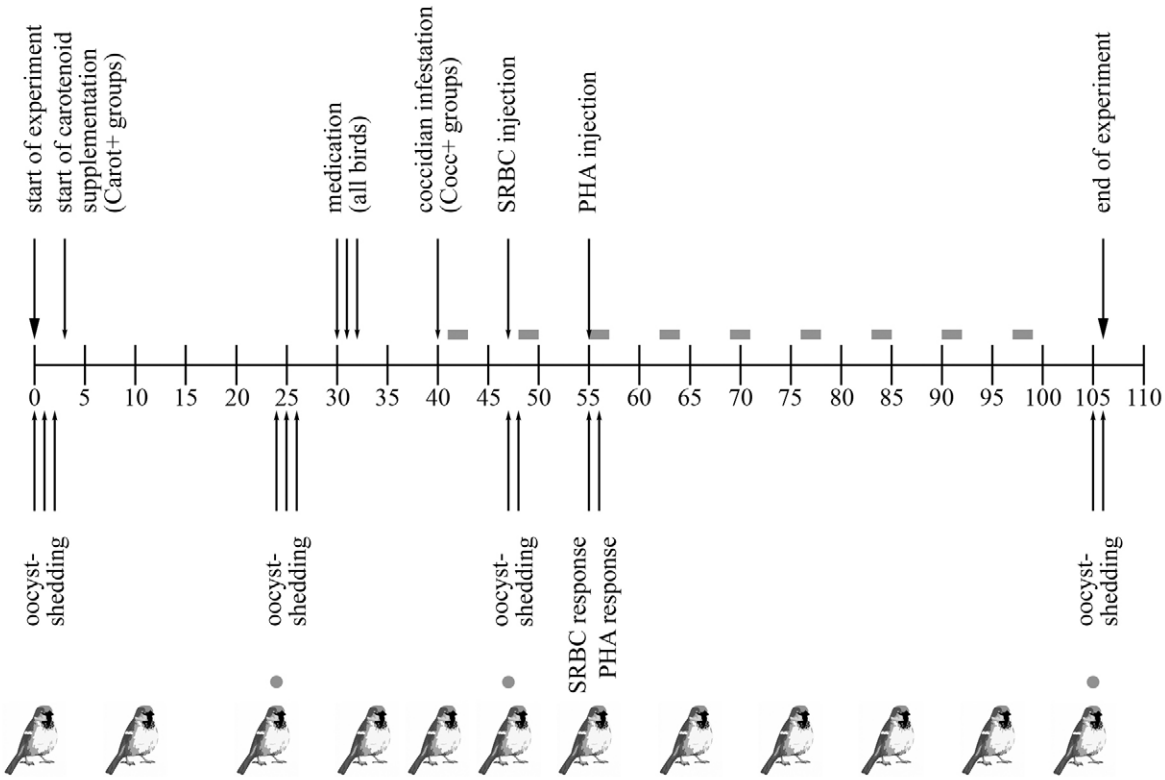

Fig. 1. Experimental protocol: day 0=28 July, day 106=9 November; above-scale narrow arrows indicate the treatment events (grey rectangles=coccidian medication in Cocc– groups; 2 days/week), and below-scale arrows the measurement events (sparrow insets=moult scoring and weighing sessions; grey circles=blood sampling for carotenoid analyses). For details see Materials and methods. Cocc– = coccidian-medicated birds; Cocc+ = coccidia-infected birds; Carot– = carotenoid non-supplemented birds; Carot– = carotenoid-supplemented.

red blood cells (SRBCs) suspension into the pectoralis muscle (see Pap et al., 2008) in order to assess the humoral immune response against a novel antigen. Because of the limited amount of blood that can be taken from a bird, we decided to use blood samples collected on the day of SRBC injection for carotenoids analyses instead of analysing the pre-immunisation antibody titre, as our previous studies conducted on the same house sparrow population had shown no detectable anti-SRBC titre under natural conditions (Pap et al., 2008). On the same day as antibody injection (day 47), the coccidian infestation was measured for the third time by moving the birds into individual cages for two days. We decided to collect faeces for only two days during the last two collection sessions compared with the three-day collection protocol used during the first and second sampling, because the number of oocysts collected from the same individual during the first two days was highly repeatable (see below), permitting an accurate estimation of the level of infection based on sampling the birds only for two days. In addition, by shortening the time spent in individual cages we could reduce the stress caused by handling and faeces collection. Eight days following the SRBC injection (day 55) we collected blood samples from the birds in order to measure the antibody titre against the SRBCs. Following blood collection we injected birds with 100 µl of 1 mg 1 ml^{-1} phytohemagglutinin (PHA) solution into the left wing web in order to measure the T-cell mediated immune response (Martin et al., 2006; Tella et al., 2008). Twenty-four hours later we measured the swelling response of birds to PHA with a pressure sensitive calliper. We collected a blood sample for carotenoid measurements when all of the birds had finished moulting (day 105), and the coccidian infestation was measured again during a two-day (105–106) faeces collection procedure. During capturing occasions (days 0, 10, 23, 33, 40, 47, 55, 65, 75, 85, 95, 104) (Fig. 1), we measured the body mass of the birds, and the moulting stage of the primaries was assessed according to the following scheme: we scored the dropped feathers as 1, a quarter-, half- or three-quarter-regrown feathers as 2, 3 and 4, respectively, and the fully-regrown feathers as 5 (for details, see Pap et al., 2008). Old feathers received score 0. For each measuring session the moulting stage of birds was characterised by the moulting score, which is the sum of the scores of individual primary feathers (total 9, the tenth is rudimentary). The moulting score ranges from 0 (before the beginning of moult: all primaries old) to 45 (moult finished: all primaries completely replaced).

Oocysts collection and experimental infestation

The coccidians were determined based on the external morphology of sporulated oocysts. By far, the specimens of *Isospora lacazei* were the most common coccidians in the faecal samples. Besides this species several unidentified oocyst types were also observed but in very low quantities (less than 5%; A.T., unpublished). Because coccidian parasites are known to be highly host specific (Greiner, 2008), we assumed that all birds were infected with the same coccidian species. As coccidians of the genus *Isospora* shed oocysts predominantly during the late afternoon (Brawner and Hill, 1999), faeces were collected just before sunset. A sheet of white paper was placed on the floor of the individual cages two hours before sunset and the faeces produced until evening were collected in tubes. After collection, the samples were stored for 2–7 days at 4°C until the laboratory analyses (see Hõrak et al., 2004). Faecal samples were

weighed to the nearest 0.01 g, suspended in 1 ml water and held at room temperature for 30 min. The solution was then drained and centrifuged at *ca.* 1800 ***g*** for 7 min. The supernatant was removed and 0.5 ml saturated NaCl water solution was added to the 0.5 ml residue. The number of oocysts was counted in 0.15 ml solution using the McMaster chamber and their concentration was expressed as the number of oocysts g^{-1} of faeces sample. The mean values of the oocysts' number collected during the two- and three-days sessions were used in the analyses. Repeatability of the infection intensity between faecal samples collected during the three days at the first collection was high (R=0.86, $F_{1,50}$=18.47, P<0.0001) and was similar to the repeatability of the data of the first two collections (R=0.86, $F_{1,50}$=14.93, P<0.0001). *Isospora lacazei* oocysts used for oral infection were collected during the first three days of the birds in the aviaries. The oocysts were sporulated in 2.5% potassium dichromate at room temperature for two days, after this time they were washed with water. On day 40 birds in Cocc+ groups were inoculated orally with approximately 2000 sporulated oocysts diluted in 2×100 µl physiological solution (see Hõrak et al., 2004). Control birds (Cocc–) received the same amount of physiological solution.

Plasma carotenoids analyses

Total carotenoids were extracted by treating 20 µl plasma with 180 µl absolute ethanol. The samples were vigorously mixed in a vortex and then centrifuged for 10 min at 6200 ***g*** for protein precipitation. Total carotenoids were determined by reading the absorbance at 450 nm with a BioTek Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA). Carotenoids concentration was calculated using a standard curve of pure lutein in the range of 1–25 µg ml^{-1} (R=0.9987) and taking in account the dilution factor of plasma. Nine plasma samples collected from wild birds were analysed using a Shimadzu LC20 AT high performance liquid chromatograph (HPLC) (Shimadzu Corp., Kyoto, Japan) with a SPD-M20A diode array detector (Shimadzu Corp.). A Hibar 250-4 Lichrosorb RP18 column (5 µm) was used (Merck KGaA, Darmstadt, Germany). The mobile phase consisted of mixtures of acetonitrile:water (9:1, v/v) with 0.25% triethylamine (solvent A) and ethyl acetate with 0.25% triethylamine (solvent B). The gradient started with 15% B, increased up to 60% B at 16 min and continued isocratically up to 25 min. The flow rate was 1 ml min^{-1} and the chromatogram was monitored at 450 nm. Lutein (39.8% of the total carotenoids), zeaxanthin (24.8%) and β-cryptoxanthin (7.8%) were identified as major carotenoids in the plasma. Quantitative data obtained by HPLC was highly positively correlated with the spectrophotometric determination (Pearson correlation, r=1.00, N=9, P<0.0001).

Measurements of anti-SRBC titre

Following blood collection in heparinised capillaries, the plasma was separated by centrifugation at 6200 ***g*** for 10 min and preserved at –20°C until further analyses. Antibody titres were measured using a base-2 serial dilution haemagglutination test conducted with 15 µl of heat-inactivated plasma (30 min on 56°C) on U-shaped 96-well microtitre plates. Samples were serially diluted starting with 15 µl PBS, and to each well 15 µl of a 1% suspension of SRBC in PBS was added. Plates were incubated at 37°C for 1 h. Titres are given as the $\log_2$ of the reciprocal of the highest dilution of plasma showing positive haemagglutination (for details, see Pap et al., 2008).

Statistical analyses

The effect of experimental manipulation on plasma carotenoids concentration, coccidian infestation, body mass, duration of moulting, and humoral and cellular immune response was analysed using general linear models (GLMs), where the dependent variables were entered separately in the model and the experimental groups were set as factors. Primary moult increases following a non-linear S-shaped function, which is very similar to the growth pattern of body mass of the developing birds. In order to describe the moulting pattern we fitted a logistic growth curve to each bird separately (Ricklefs, 1973), a model used for study of avian growth. The logistic growth curve has a form of $y=a/\{1+e^{[-K(t-I)]}\}$, where y denotes moulting score of a bird at time t, a is the final score or asymptote, which has a fixed value of 45 in our case, K is the growth constant, I is the inflection point on the time axis in which moulting changes from accelerating to decelerating and e is the base of natural logarithm. Following the calculation of moulting parameters we tested the effect of coccidian infestation and carotenoid supplementation on moulting speed (K) and inflection point (I) using a two-way analysis of variance (ANOVA) test. All data on infestation were log(x+1)-transformed in order to normalise the skewed distribution of the number of oocysts. Because birds captured from the two farms differed in their initial infestation rate ($F_{1,49}$=42.3, P<0.0001), we statistically removed the effect of farms by using the residuals of the initial log number of oocysts from the GLMs, where the place was entered as an explanatory factor. Birds originating from different farms were distributed randomly and evenly between the experimental groups. Besides initial infestation rate no difference was found between birds from the two farms at capture in wing length ($F_{1,49}$=0.88, P=0.35), tarsus length ($F_{1,49}$=0.65, P=0.42) and body mass ($F_{1,49}$=1.31, P=0.26). Sample sizes vary among analyses because in certain samplings some birds have missing or incomplete data (no faeces, low-quantity of plasma, dead birds). Means ± s.d. are shown throughout the text unless otherwise stated.

RESULTS

The effect of treatments on coccidian infestation and plasma carotenoids concentration

Before the experiment started (day 0) there was no difference between experimental groups in the residual intensity of infestation of birds with oocysts (Carot: $F_{1,47}$=0.01, P=0.92; Cocc: $F_{1,47}$=0.01, P=0.93; Carot × Cocc interaction: $F_{1,47}$=1.22, P=0.28) (Fig. 2). Carotenoid supplementation had no significant effect on the natural coccidian infestation rate of the birds on day 24 ($F_{1,49}$=0.02, P=0.88). The experimental infestation with coccidians resulted in a significant difference in oocyst-shedding rate between infestation groups on day 47 ($F_{1,46}$=37.17, P<0.0001) (Fig. 2) whereas carotenoid supplementation had no significant effect ($F_{1,46}$=0.25, P=0.62; Carot × Cocc interaction: $F_{1,46}$=0.25, P=0.62). The effect of infestation treatment on the level of coccidian infestation of the birds persisted until the end of the experiment (day 105), as the difference between infestation groups remained significant ($F_{1,45}$=54.12, P<0.0001), while the effect of carotenoid supplementation was again not significant ($F_{1,45}$=0.08, P=0.78; Carot × Cocc interaction: $F_{1,45}$=0.93, P=0.34).

Carotenoid supplementation increased the plasma carotenoids concentration before infestation at day 24 ($F_{1,45}$=14.91, P=0.0004) (Fig. 3) whereas no significant difference was found between coccidian groups, as expected ($F_{1,45}$=0.64, P=0.43; Carot × Cocc interaction: $F_{1,45}$=0.20, P=0.65). Following infestation, on day 47, the difference between carotenoid groups persisted ($F_{1,45}$=14.25, P=0.0005), and the plasma carotenoids concentration was similar between infested and medicated groups ($F_{1,45}$=1.72, P=0.20; Carot × Cocc interaction: $F_{1,45}$=0.49, P=0.49). At the end of the

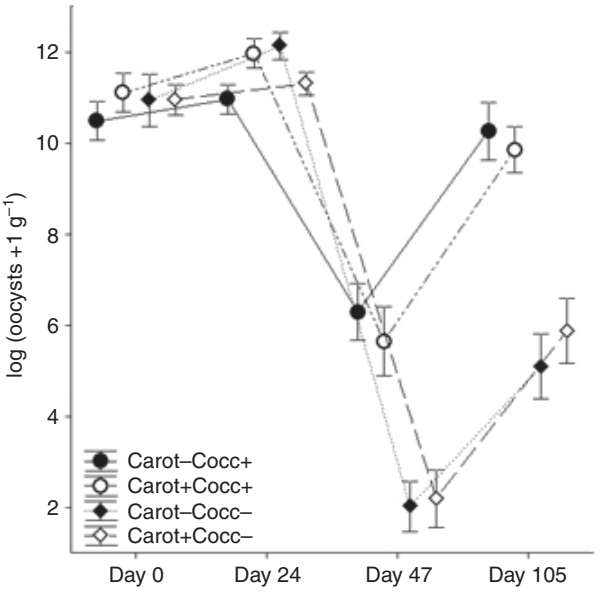

Fig. 2. Change in oocyst-shedding (means ± s.e.m.) in different groups during the course of the experiment. House sparrows were introduced in the aviaries on 24 and 26 July and the carotenoid manipulation started on day 3 (day 0=28 July). All birds were medicated for three days starting on day 30, followed by the re-infection of house sparrows from Cocc+ groups on day 40. Cocc– = coccidian-medicated birds; Cocc+ = coccidia-infected birds; Carot– = carotenoid non-supplemented birds; Carot– = carotenoid-supplemented.

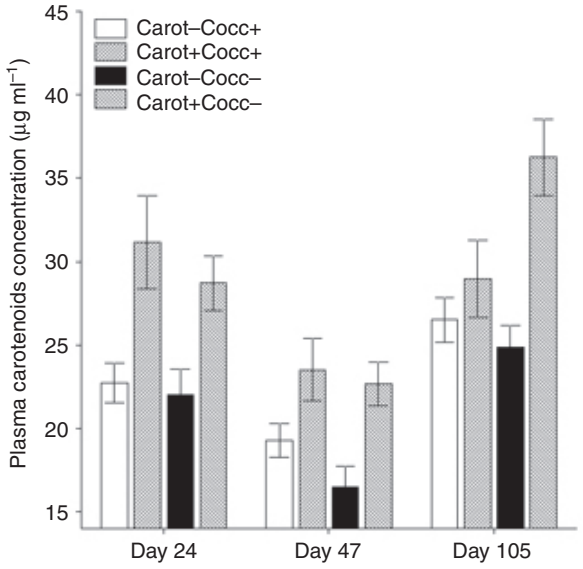

Fig. 3. Change in plasma carotenoids concentration between groups during the course of the experiment. Bars represent means ± s.e.m. Cocc– = coccidian-medicated birds; Cocc+ = coccidia-infected birds; Carot– = carotenoid non-supplemented birds; Carot– = carotenoid-supplemented.

experiment (day 105), the difference in carotenoid concentration between carotenoid groups remained significant ($F_{1,45}$=13.37, P=0.0007), and the effect of coccidian infestation was not significant ($F_{1,45}$=2.25, P=0.14). However, the increase was significantly larger in the Carot+Cocc– group compared with the rest of the experimental groups (Fig. 3), as indicated by the significant Carot × Cocc interaction ($F_{1,45}$=5.53, P=0.023). No difference was found in plasma carotenoid concentration between Carot–Cocc+, Carot+Cocc+ and Carot–Cocc– groups (Tukey *post hoc* test, P>0.43 in all cases). Natural coccidian infestation and plasma carotenoids concentration measured at day 24 were marginally significantly and negatively or not significantly related within Carot+ (r=–0.39, N=26, P=0.051) and non-significantly in Carot– groups (r=0.15, N=23, P=0.51). Following infestation of the Cocc+ birds, coccidian infestation and plasma carotenoids concentration, measured on days 47 and 105, were not, or were marginally, significantly and negatively correlated within Carot+ (day 47: r=–0.44, N=12, P=0.15; day 105: r=0.16, N=13, P=0.60) and Carot– groups (day 47: r=–0.38, N=12, P=0.22; day 105: r=–0.56, N=12, P=0.06).

The effect of treatments on condition and moult

Experimental infestation with coccidians and carotenoid supplementation had a significant effect on the change in body mass of the birds during seven days following infection (Table 1; Fig. 4). Body mass of the birds from the Carot–Cocc+ group decreased more sharply compared with the Carot+Cocc+ group (Tukey *post hoc* test, P=0.006), and the decrease tended to be smaller in the Carot+Cocc– group compared with the Carot–Cocc– group (P=0.06). The difference between experimental groups disappeared 15 days following infection (Table 1).

There was no difference between experimental groups at the start of moulting (GLM with binomial distribution of the dependent variable; Carot: $\chi^2_{1,47}$=0.02, P=0.90; Cocc: $\chi^2_{1,47}$=0.20, P=0.66; Carot × Cocc interaction: $\chi^2_{1,47}$=1.04, P=0.31). Experimental manipulation had no significant effect on the speed of moult on the seventh and fifteenth days following infection (Table 1). Experimental infestation with coccidians had no significant effect on the moulting parameters measured during the whole moulting period (K value: $F_{1,45}$=0.07, P=0.79; I value: $F_{1,45}$=0.00, P=0.97), and the effect of carotenoid supplementation was not significant on the K value ($F_{1,45}$=1.00, P=0.32) and had a positive effect on I of moulting ($F_{1,45}$=6.64, P=0.01). None of the interactions between experimental groups was significant (K value: $F_{1,45}$=0.26, P=0.62; I value: $F_{1,45}$=0.16, P=0.69). The duration of moult (calculated as the time elapsed between the first and the last measuring sessions, namely when a dropped innermost primary was observed first and

Table 1. General linear models (GLMs) about the effect of experimental treatments of coccidian infection (factor) and carotenoid supplementation (factor) on the change in body mass and speed of moult seven and 15 days following infection

Source	$F_{d.f.}$	P
Change in body mass 7 days following infection		
Coccidian infestation	$12.35_{1,46}$	0.001
Carotenoid supplementation	$20.02_{1,46}$	0.0001
Coccidian infestation × carotenoid supplementation	$0.54_{1,46}$	0.47
Change in body mass 15 days following infection		
Coccidian infestation	$0.02_{1,46}$	0.90
Carotenoid supplementation	$1.72_{1,46}$	0.20
Coccidian infestation × carotenoid supplementation	$0.43_{1,46}$	0.51
Increase in moulting score 7 days following infection		
Coccidian infestation	$0.30_{1,46}$	0.59
Carotenoid supplementation	$1.05_{1,46}$	0.31
Coccidian infestation × carotenoid supplementation	$0.30_{1,46}$	0.59
Increase in moulting score 15 days following infection		
Coccidian infestation	$0.00_{1,46}$	0.99
Carotenoid supplementation	$0.59_{1,46}$	0.45
Coccidian infestation × carotenoid supplementation	$0.08_{1,46}$	0.77

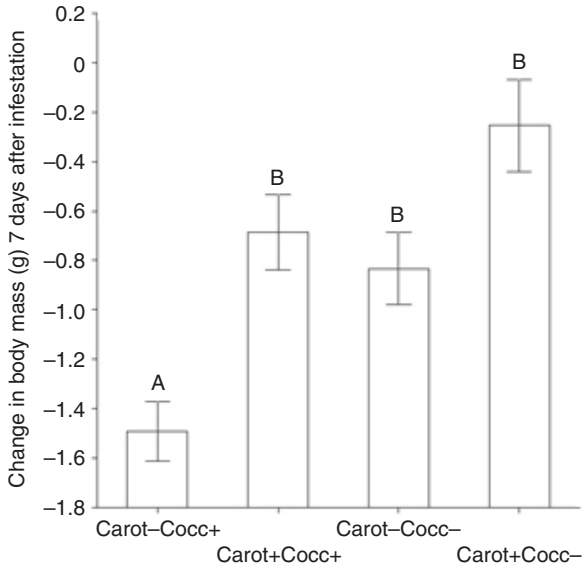

Fig. 4. The effect of coccidian infestation on the change in body mass seven days following infestation in experimental groups. Bars represent means ± s.e.m.; letters indicate group membership resulting from Tukey *post hoc* test (see text). Cocc– = coccidian-medicated birds; Cocc+ = coccidia-infected birds; Carot– = carotenoid non-supplemented birds; Carot– = carotenoid-supplemented.

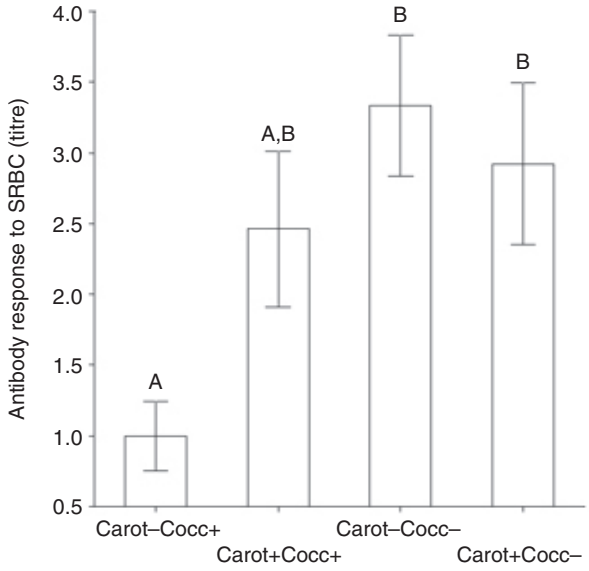

Fig. 5. The effect of experimental manipulation on the strength of antibody response against sheep red blood cells (SRBCs). Bars represent means ± s.e.m.; letters indicate group membership resulting from Tukey *post hoc* test (see text). Cocc– = coccidian-medicated birds; Cocc+ = coccidia-infected birds; Carot– = carotenoid non-supplemented birds; Carot– = carotenoid-supplemented.

when all primary feathers were fully grown) was similar between experimental groups (Carot: $F_{1,45}$=0.47, P=0.50; Cocc: $F_{1,45}$=1.34, P=0.25; Carot × Cocc interaction: $F_{1,45}$=0.03, P=0.87).

The effect of treatments on immune responses

Coccidian infestation significantly and negatively affected the capacity of the birds to respond against the SRBC injection on day 55 ($F_{1,46}$=8.08, P=0.007) (Fig. 5), while the effect of carotenoid supplementation was not significant ($F_{1,46}$=1.14, P=0.29). The near significant Carot × Cocc interaction ($F_{1,46}$=3.62, P=0.06) indicates that carotenoid supplementation increased the immune response of birds from the infested groups but had no effect within the medicated groups (Fig. 5). Within the Carot–Cocc+ group, the antibody titre correlated significantly and positively with the infestation rate measured during SRBC injection on day 47 (r=0.65, N=12, P=0.023) (Fig. 6A) whereas in the Carot+Cocc+ group the relationship was not significant (r=–0.40, N=13, P=0.18) (Fig. 6B). In none of the experimental groups was the SRBC antibody response related to the plasma carotenoids concentration measured during antigen injection on day 47 (Carot–Cocc+: r=–0.19, N=*12*, P=0.56; Carot+Cocc+: r=0.36, N=12, P=0.25; Carot–Cocc–: r=0.03, N=12, P=0.92; Carot+Cocc–: r=0.13, N=13, P=0.67). The PHA inflammation immune response of the birds was not affected by coccidian infestation ($F_{1,46}$=0.35, P=0.56) and carotenoid manipulation ($F_{1,46}$=0.00, P=0.69; Carot × Cocc interaction: $F_{1,46}$=1.54, P=0.22), and within the infected groups no significant relationship was found between the magnitude of swelling and infestation rate measured prior to injection on day 47 (Carot–Cocc+: r=–0.48, N=12, P=0.12; Carot+Cocc+: r=–0.02, N=13, P=0.94). PHA swelling was not related to the plasma carotenoids concentration measured on day 47 in any of the experimental groups (Carot–Cocc+: r=0.28, N=37, P=0.56; Carot+Cocc+: r=–0.04, N=12, P=0.89; Carot–Cocc–: r=0.57, N=12, P=0.054; Carot+Cocc–: r=0.02, N=13, P=0.94).

DISCUSSION

In the present study we found that experimental infestation of moulting house sparrows with coccidians had a significant but transient negative effect on the body mass related to the medicated groups, and the negative effect of coccidians on the change in body mass during seven days following infection was balanced by carotenoid supplementation. We found no difference in the change in body mass following infestation between Carot+Cocc+, Carot–Cocc– and Carot+Cocc– groups, indicating that birds were able to fully compensate the negative effect of infestation if carotenoids were added to their food, as predicted. This finding suggests that the negative effect of coccidians on condition is modulated by the availability of carotenoids in the diet, supporting the results of previous studies about the role of these biomolecules in mediating the trade-off between defence and reproduction in birds and fish (Kolluru et al., 2006; Baeta et al., 2008). The mechanisms behind the neutralising action of carotenoids can be explained by the immunostimulating and immunoregulating effects of these pigments (reviewed by Møller et al., 2000; Catoni et al., 2008), which may play an important role in the defence against parasites. Most of the previous studies, where the availability of carotenoids was experimentally manipulated, indicate that increased availability affects the plasma carotenoids concentration and stimulates the immune response of birds and fish (Blount et al., 2003; McGraw and Ardia, 2003; Saino et al., 2003; Grether et al., 2004; Peters et al., 2004; Cucco et al., 2006; Hõrak et al., 2006; Kolluru et al., 2006; McGraw et al., 2006; Aguilera and Amat, 2007) [but see Navara and Hill (Navara and Hill, 2003) and Smith et al. (Smith et al., 2007)]. This is exactly what we also found, i.e. within the experimentally infested groups, birds with carotenoid-supplemented food tended to have an increased humoral immune response to SRBC compared with non-supplemented birds. Interestingly, we found no impact of carotenoid supplementation on the immune function of birds from the medicated groups, suggesting that the

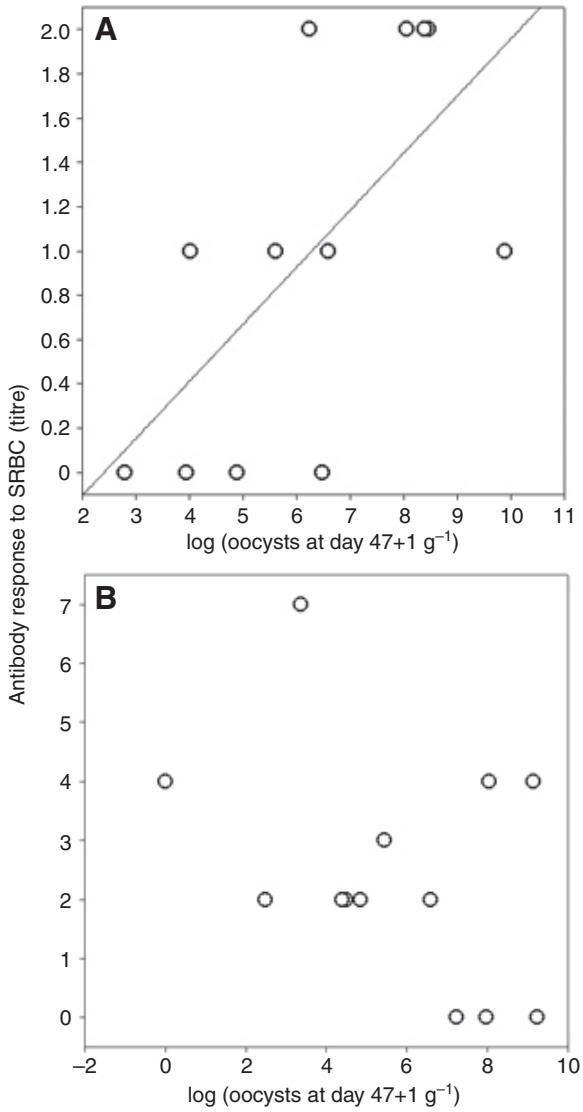

Fig. 6. The relationship between antibody response against sheep red blood cells (SRBCs) and coccidian infestation measured at the day of injection (day 47) in (A) Carot–Cocc+ and (B) Carot+Cocc+ groups. The regression line in A is y=0.26x–0.62. Cocc– = coccidian-medicated birds; Cocc+ = coccidia-infected birds; Carot– = carotenoid non-supplemented birds; Carot– = carotenoid-supplemented.

immunostimulant properties of these pigments act only under parasite pressure when the immune system is stimulated. Further, the positive correlation between coccidian infestation and the strength of the humoral immune response against SRBC in the Carot–Cocc+ group indicates that this part of the immune system plays an important role in the defence against these parasites (see Saks et al., 2006). Carotenoids may modulate the effect of coccidian infestation on immune defence, as within the infected and supplemented group no relationship was found between parasitism and antibody titre. This can be explained by the disproportionately positive effect of carotenoids on humoral immune response in birds with initial low antibody titre. It is interesting to note that, contrary to our expectations based on previous studies (Kolluru et al., 2006; Baeta et al., 2008) and on the increased immune function of carotenoid-supplemented birds compared with the non-supplemented group, we have found no effect of carotenoid supplementation on the intensity of coccidian parasitism on birds. This result is in line with the study by Navara and Hill, where the experimental carotenoid supplementation had no significant effect on the mycoplasmal conjuctivities in the American goldfinches (*Carduelis tristis*) (Navara and Hill, 2003).

In contrast to our results about SRBC immune response, the PHA swelling was not improved by carotenoid supplementation and experimental coccidian infestation. Furthermore, we found no significant relationship between the strength of the swelling response to PHA and coccidian infestation. These findings suggest that carotenoids might have differential effects on the separate components of the immune system, and immune variables may have diverse roles in the defence against coccidians. Interestingly, the effect of carotenoid supplementation on immune responses vary between studies conducted on various avian species where different immunological tests were used. For example, carotenoids may have positive effect on cellular immunity (Blount et al., 2003; McGraw and Ardia, 2003; Saino et al., 2003; Cucco et al., 2006), on humoral immune response (McGraw and Ardia, 2003; Aguilera and Amat, 2007), on innate immune function (McGraw et al., 2006) or on none of the variables measured (Navara and Hill, 2003; Smith et al., 2007). Our results, in line with previous studies, suggest the inherent variability of birds in their carotenoid-dependent immune function. Note, however, that there are only some species with elaborate carotenoid-based plumage colouration, where carotenoids seem to have no effect on at least some components of the immune system (Navara and Hill, 2003; Smith et al., 2007).

The role of carotenoids in determining the plumage colouration of birds and hence its importance in sexual selection is well established (Hill and McGraw, 2006a; Hill and McGraw, 2006b). However, McGraw et al. (McGraw et al., 2006) and the present study indicate that the absence of carotenoid-based plumage and skin traits is not a requirement for carotenoid dependency of the immune system in birds. Carotenoids may be important in these species through at least two physiological pathways. First, through a general immunoregulation effect, carotenoids may buffer the negative effects of testosterone on the immune system (Folstad and Karter, 1992) during critical states, like mating. In this case, individuals with high carotenoid reserves may have superior immune function even under the immunosuppressive effect of testosterone. Second, carotenoids may have a positive effect on the sexual and parental activity of birds, as suggested by the study of Blount and Matheson, where the carotenoid supplementation enhanced the flight performance of the zebra finches (*Taeniopygia guttata*) (Blount and Matheson, 2006).

Because coccidians, like other intestinal parasites may constrain the uptake of carotenoids from the alimentary canal (Hõrak et al., 2004; Martínez-Padilla et al., 2007; Mougeot et al., 2007; Baeta et al., 2008; Mougeot et al., 2009), we would expect the plasma carotenoid concentration to decrease in the experimentally infested birds compared with the medicated groups. Our results show that seven days following infestation (day 47), the change in plasma carotenoid concentration is similar between Carot+Cocc+ and Carot+Cocc–, and between Carot–Cocc+ and Carot–Cocc– groups. The lack of a relationship between infestation and plasma carotenoid concentration can be explained by the relatively short period elapsed following infestation, meanwhile the intensity of coccidians could not reach a threshold level to inhibit the absorption of carotenoids in the alimentary canal. After prolonged (65 days) exposure to coccidians, at the end of the experiment (day 105), the plasma carotenoid concentration was similar between the Carot+ Cocc+, Carot–Cocc+ and Carot–Cocc– groups and was highest in the

Carot+Cocc– birds. This suggests that only those birds could utilise the supplemented carotenoids in the diet that were released from the pressure of infestation by coccidians. Interestingly, despite our *a priori* expectation about the reduced plasma carotenoids concentration in the Carot–Cocc+ compared with Carot+Cocc+ birds, we found no significant difference between groups even after a prolonged exposure to coccidians. One possible explanation for this result is the mobilisation of carotenoid reserves from deposits like liver and fat in Carot–Cocc+ birds in order to maintain the plasma carotenoids concentration, which are important for stimulating the immune system. Alternatively, but not exclusively, supplemented and infested birds utilised the carotenoid surplus to upregulate their immune system (Pérez-Rodríguez et al., 2008), as supported by the increased humoral immune function of Carot+Cocc+ compared with Carot–Cocc+ birds.

In summary, we have shown that carotenoids may have an important role in mediating the effect of coccidian parasitism on condition in the moulting house sparrow. The relationship between parasitism, carotenoids and susceptible traits is realised through the immune system, as suggested by the immunostimulating effect of carotenoids and the significant positive correlation between antibody response to SRBC and coccidian infestation. This suggests that carotenoids are important micronutrients in maintaining the physiological homeostasis not only in species with carotenoid dependent characters but also in birds with no carotenoid-based signals.

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