Carotenoid trade-off between parasitic resistance and sexual display: an experimental study in the blackbird (*Turdus merula*)

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Many parasites depress the expression of the carotenoid-based colour displays of their hosts, and it has been hypothesized that animals face a trade-off in carotenoid allocation between immune functions and 'degree of ornamentation'. While numerous correlative studies suggest that parasite infection decreases the intensity of carotenoid-based colour displays, the existence of this trade-off has never been demonstrated experimentally in a host–parasite model. In this study, we used the blackbird (*Turdus merula*) and *Isospora* (an intestinal parasite) to assess whether this trade-off does indeed exist. Blackbirds were supplemented with carotenoids while simultaneously being exposed to parasites. Supplemented males circulated more carotenoids in the blood and developed more brightly coloured bills than unsupplemented males. In addition, supplementation slowed down the replication rate of parasites. Supplementation with carotenoids enabled infected birds to maintain their bill coloration, whereas birds that were infected but not supplemented showed reduced bill coloration. At the same time, infection slowed carotenoid assimilation in the blood. Overall, we demonstrated that bill colour reflects a bird's health, and that only males with a carotenoid-rich diet are capable of coping with costs associated with parasitic infection. Carotenoids are thus traded off between host physiological response to parasites and secondary sexual traits. Further investigations are required to determine the physiological mechanisms that govern this trade-off.

Keywords: bill colour; carotenoids; coccidia; experimental infection; trade-off

1. INTRODUCTION

In many species, carotenoid-based coloration is more elaborate in males than in females, and carotenoid-based sexual traits have been shown to be involved in the mate-choice process (Endler 1983; Burley & Coopersmith 1987; Hill 1990, 1991, 1994). The mechanisms ensuring the honesty of carotenoid-based sexual traits have been extensively studied, and among the hypotheses that have been put forward to explain signal honesty is that carotenoid-based traits are maintained by a trade-off between the ornamentation and the immune/detoxification system (Blount *et al.* 2003; Faivre *et al.* 2003; McGraw & Ardia 2003). Indeed, carotenoids are known to enhance immune defences (Bendich 1989; Chew 1993; Jyonouchi *et al.* 1994; Møller *et al.* 2000) and scavenge free radicals and cytotoxic molecules produced during daily metabolic processes and immune reactions (Burton 1989; Møller *et al.* 2000). If carotenoids are required for several functions at the same time (Fitze *et al.* 2007) and if they represent a limited resource, they should then be traded off between self-maintenance and secondary sexual ornamentation (Lozano 1994). Consequently, when male self-maintenance is threatened by parasites, carotenoids should be allocated to fight the infection at the expense

of the expression of secondary sexual ornamentation. However, it is also possible that the parasites themselves may alter the carotenoid allocation process, and that the classic view of the allocation trade-off has been challenged by findings showing that immune activation may modify carotenoid absorption and/or transportation (McGraw *et al.* 2005; Fitze *et al.* 2007).

Many correlative studies have supported this trade-off hypothesis and have reported negative relationships between carotenoid-based coloration and parasitism in both fishes and birds (Milinski & Bakker 1990; Zuk *et al.* 1990; Houde & Torio 1992; Thompson *et al.* 1997; Brawner *et al.* 2000; McGraw & Hill 2000; Møller *et al.* 2000). In addition, previous experimental studies using parasites have found that (i) controlled infections reduce the expression of carotenoid-based ornamentation (Hill & Brawner 1998; Brawner *et al.* 2000; Horak *et al.* 2004), (ii) parasite eradication positively affects carotenoid-based ornamentation (Martinez-Padilla *et al.* 2007), and (iii) expression level of ornaments may indicate males' ability to clear parasite infection (Lindström & Lundström 2000). Furthermore, newly developed methods, which allow measurement of the magnitude of the immune response to various antigens (see Adamo (2004) for a review), made it possible to identify a positive relationship between carotenoid-based coloration and various immune effectors (Blount *et al.* 2003; McGraw & Ardia 2003 and references therein), and have also shown that immune defence and sexual attractiveness are constrained by

carotenoid availability (Blount *et al.* 2003; Grether *et al.* 2004; McGraw & Ardia 2004). However, the potential link between parasitic infection and expression of secondary sexual traits needs further investigation because both parasites themselves and parasitic resistance may affect ornamentation in different ways. As emphasized by Griffith *et al.* (2006), a valuable way to test a trade-off between resistance to parasite and colour is to vary both the level of carotenoids provided and the level of parasitic infection at the same time, which allows for a simultaneous assessment of the role of the pigment in self-maintenance and the potential trade-off in allocation to either parasitic resistance or signalling. To our knowledge, only one study has attempted such an experiment, through the carefully controlled exposure of American goldfinches (*Carduelis tristis*) to the parasite *Mycoplasma gallisepticum* (Navara & Hill 2003). With this design, manipulations to both carotenoid availability and infection status were carried out simultaneously, allowing useful inferences to be made about the direct role of carotenoid availability in any trade-off (Fitze *et al.* 2007). However, this study failed to find the evidence of a trade-off between colour and response to infection.

In this study, we experimentally investigated whether carotenoids are traded off between sexual displays and parasite resistance in the blackbird *Turdus merula*. In this species, male bill colour varies from pale yellow to orange (Cramp *et al.* 1988) due to carotenoids (Faivre *et al.* 2003), and several studies have suggested that this trait is sexually selected (Faivre *et al.* 2001; Bright & Waas 2002; Bright *et al.* 2002). Blackbirds are naturally infected with intestinal parasites from the genus *Isospora* Protozoa, Apicomplexa (Misof 2004). These protozoans have both sexual and asexual phases. In most species, the asexual phase occurs in the intestinal epithelium of the host, where one or more asexual multiplication cycles are completed before sexual development occurs (Olsen 1974). Oocysts produced during the sexual phase are released into the host's intestinal tract and passed in the faeces. Birds are orally infected when ingesting sporulated oocysts released in the faeces of an infected host.

In this experiment, blackbirds were supplemented with immunoenhancing carotenoids (lutein and zeaxanthin, Kim *et al.* 2000; Blount *et al.* 2003; McGraw & Ardia 2003 but see Navara & Hill 2003; Fitze *et al.* 2007) for four weeks. Preliminary studies in blackbirds have shown that males supplemented with these two pigments produced higher cell-mediated immune responses than controls (J. Moreau 2005, unpublished results). At the same time, blackbirds were exposed to an experimental infection with sporulated oocysts of *Isospora* sp. Our aim with this procedure was to test the role of carotenoid availability in trade-offs. Although our design does not allow us to identify the exact mechanism linking carotenoids, parasites, immune response and free radical scavenging, it may help us to tease apart several competing hypotheses on the negative effect of infection on carotenoid-based signals. For example, if parasites reduce carotenoid allocation to a sexual signal, we would expect that infection should negatively affect blackbird bill colour. Further, if carotenoids are traded off between sexual display and parasitic resistance, bill colour should be less affected in birds supplemented with carotenoids, if at all.

2. MATERIAL AND METHODS

(a) *General procedure*

Forty-four adult male blackbirds (at least 2 years old) were caught using mist nets in urban parks in Dijon (France, $47°19'$ N, $5°02'$ E) from January to March 2005. After capture, birds were kept in outdoor aviaries ($220 \times 150 \times 250$ cm), fed ad libitum with food for large turdid species (COFNA) and given tap water throughout their time in captivity. Water, carotenoids and food were replaced each day.

During the four-week experimental period, from mid-April (day 1) to mid-May (day 28), birds were kept on a natural light cycle. One week before starting the experiment, all birds were placed in individual outdoor cages ($69.5 \times 44.5 \times 82.5$ cm) and fed using the same regimes.

Forty-four male blackbirds were randomly assigned to one of the four treatment groups based on carotenoid supplementation and parasite exposure. Twenty-two birds supplemented with carotenoids (C+) were provided 150 μg ml^{-1} of carotenoids daily diluted in drinking water (Oro Glo liquid, 11 mg ml^{-1}, lutein and zeaxanthin [20 : 1, w/w]; Kemin France SRL, Nantes), while the remaining birds (C−) were provided tap water only. The diet of blackbirds is extremely variable and depends on time and location (Snow 1958; Cramp *et al.* 1988). To our knowledge, no data are available that quantify carotenoid content of the blackbird diet. Therefore, we calibrated the dose according to the study of Alonso-Alvarez *et al.* (2004) on zebra finches (*Taeniopygia guttata*). This dose is included in the range generally used for supplementation in previous studies (e.g. Hill 1992; McGraw *et al.* 2001; Blount *et al.* 2003; Navara & Hill 2003). Liquids were provided in opaque dispensers to avoid oxidation and protect the carotenoids from light (see Blount *et al.* 2003). Half of the birds (randomly selected) in each treatment group were inoculated with isosporan oocysts (see procedures below), while the other half acted as the controls, and were given water only. Inoculation was conducted using a cannula that was gently introduced in the throat to reach the oesophagus.

One week before the experiment, faecal samples were collected from all the birds to assess the baseline intensity of infection with coccidian. At this time, the level of infection was very low (fewer than 80 oocysts g^{-1} of faecal sample for each group). Preliminary observations showed that blackbirds kept in captivity almost always become uninfected, unlike greenfinches (Horak *et al.* 2004). This may have been due to the conditions in which birds were kept; aviaries were cleaned twice a week and water and food were renewed daily. Our cleaning schedule meant that infected faeces were often removed thus disrupting the parasite cycle, and preventing cross and successive infections. As a result, we did not use an anticoccidian drug to obtain uninfected birds, and thus avoided the potential confounding effects of drug use. If we had used these drugs, we could not have excluded side effects of anti-coccidian drugs on the general condition of birds and/or on physiological functions associated with carotenoid metabolism.

(b) *Procedure for the experimental infection and assessment of individual parasite load*

To obtain the isosporan oocysts needed for the experimental infection, we collected faeces from one heavily naturally infected adult blackbird male. This bird was caught two weeks before the beginning of experiments and was housed individually in an outdoor cage ($69.5 \times 44.5 \times 82.5$ cm). An aluminium sheet was placed on the bottom of the cage to collect the infected faeces. The faeces were collected daily for

3 days and placed in a solution of potassium dichromate diluted with water ($K_2Cr_2O_7$, 2.2%) for oocyst storage. The suspension was stored at 4°C. To obtain oocyst sporulation before experimental infection, a thin layer of this suspension was exposed to air for at least 5 days. Oocysts were then washed with distilled water to remove the potassium dichromate from the suspension. Four washing procedures were sequentially performed: 1 ml of the suspension of potassium dichromate was mixed with 9 ml of distilled water and centrifuged at 2500*g* for 10 min. The supernatant was removed after centrifugation and the residue containing oocysts was mixed with 9 ml of water. After the last washing procedure, the oocysts were brought to the surface by dissolving 1 ml of residue into 10 ml of a Sheater solution (a 45% sugar solution). The concentration of oocysts used to prepare the infecting solution was adjusted to 10 000 sporulated oocysts ml^{-1}. This solution was suspended in water based on the count of viable oocysts, using a McMaster chamber which allowed us to determine the density of evenly suspended particles. To do this, we deposited 600 µl of this solution in the chamber and the fully sporulated oocysts. On day 1, each male from the infected group was inoculated orally with 10 000 isosporan oocysts suspended in water, while birds from the control group were given an equal volume of water. This dose for infection was calibrated according to the dose used by Horak *et al.* (2004) in greenfinches (*Carduelis chloris*) and should be biologically meaningful in blackbirds, given our recent observations that 0.1 g of blackbirds' faeces can contain more than 10 000 oocysts (J. Moreau 2007, unpublished results).

To determine individual parasite loads, an aluminium sheet was placed on the bottom of the cage to collect faeces from 16.30 to 19.00 on days 0 (i.e. 1 day before the start of supplementation with carotenoids and the inoculation with isosporan oocysts), 7, 14, 21 and 28. Faeces were sampled in the afternoon because blackbirds infected with *Isospora* show a diurnal periodicity of oocyst elimination, with a peak in the late afternoon (as in many passerines, Brawner *et al.* 2000; Misof 2004). The collected faecal samples were weighed (± 0.01 g) with an electronic balance (Scout Pro) and placed in a 50 ml Falcon tube. We added 14 ml of a Sheater solution, gently homogenized the mixture and placed 600 µl of this solution in a McMaster chamber to count the number of floating oocysts. Two counts were conducted from individual faecal samples and the mean number of oocysts was used as the estimate of oocyst abundance. Oocyst concentration was expressed as number of oocysts per gram of faecal sample. Preliminary work showed that oocyst number assessment was highly repeatable (intra-class correlation coefficient according to Lessells & Boag (1987), $n = 20$, $r = 0.94$, $p < 0.0001$).

(c) *Blood sampling and plasma carotenoids concentration*

Blood samples were collected from each bird through the brachial vein using sterile needles and heparinized micro-capillary tubes. Blood was immediately centrifuged (4000*g*, 4°C, 15 min) and plasma was stored in 1.5 ml Eppendorf tubes at −80°C for later analysis. Total circulating carotenoid concentration was determined three times during the study: (i) on the day before carotenoid supplementation (day 1), (ii) two weeks after the initiation of treatments (day 14), and (iii) four weeks after starting the treatments (day 28). Concentration was assessed using a standardized colorimetric technique (detailed in Alonso-Alvarez *et al.* 2004). Twenty
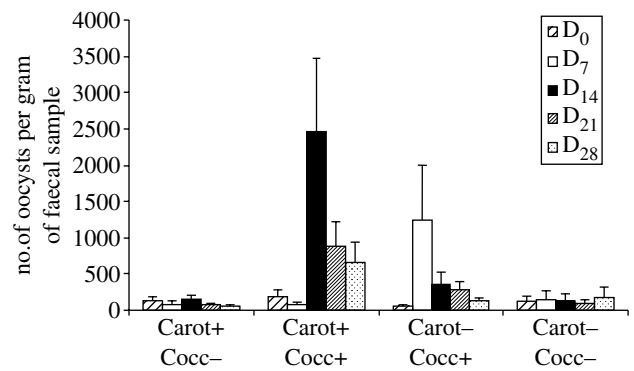
Figure 1. Number of oocysts of *Isospora* shed in male faeces (mean + s.e.) weekly from D_0 to D_{28} according to the availability of carotenoids (Carot+ for carotenoid supplemented and Carot− for no carotenoid supplemented) and their infection status (Cocc+ for experimentally infected and Cocc− for not experimentally infected).

microlitres of plasma were diluted in 180 µl of absolute ethanol, mixed with a vortex and then the flocculent proteins were precipitated by centrifuging the sample at 1500*g* for 10 min. The supernatant was analysed using a spectrophotometer to determine the optical density of the carotenoid peak at 450 nm. Carotenoid concentration was determined from a standard curve of lutein. The repeatability of this measure was high (intra-class correlation coefficient according to Lessells & Boag (1987), $n = 20$, $r = 0.96$, $p < 0.0001$).

(d) *Bill colour scoring*

Bill colour was scored on days 1, 14 and 28 at the same time as that of blood sampling. Colour was assessed following the method used by Faivre *et al.* (2001) by comparison with a coloration index derived from a Yolk Colour Fan (ROCHE, Neuilly sur-Seine, France) ranging from index 1 (pale yellow) to index 15 (orange). In a previous study, this coloration index was compared with an assessment of bill colour using a spectroradiometer and was consistent with these measurements (see Faivre *et al.* (2001) for any additional information). We chose this scoring method to reduce handling time of birds. Measurements were always taken by the same observer under the same light conditions and were highly repeatable (intra-class correlation coefficient according to Lessells & Boag (1987), $n = 20$, $r = 0.88$, $p < 0.0001$).

(e) *Body mass measuring*

Body mass was measured on days 1, 14 and 28 at the same time as other measurements. Mass was assessed using an electronic balance (Scout Pro), accurate to the nearest 0.1 g.

(f) *Statistical analysis*

The effects of experimental treatments (coccidian infection and carotenoid supplementation) on the dynamics of parasite load, bill colour, plasma carotenoid and body mass were assessed by repeated measure analysis of variance (ANOVA). The full models are presented here including all interaction terms. Assumption for the parametric analyses was met for body mass. Parasitic load, bill colour and plasma carotenoid were log transformed to attain normality. Two-tailed tests of significance were used throughout. The statistical tests were performed using the JMP software (v. 3.2.2, SAS institute, Inc.).

Table 1. Results of repeated measure analysis of variance on dynamics of parasite load, bill colour, plasma carotenoids and body mass of male blackbirds. (Directions of the effects are presented in figures 1 and 2. Cocc, isosporan experimental infection; Carot, carotenoid supplementation.)

	change on life-history traits							
	(a) parasite load		(b) bill colour		(c) plasma carotenoids		(d) body mass	
effect test	F	p	F	p	F	p	F	p
between subjects								
Cocc	$F_{1,40}=9.42$	*0.004*	$F_{1,40}=1.85$	0.18	$F_{1,40}=2.44$	0.13	$F_{1,40}=0.13$	0.72
Carot	$F_{1,40}=0.03$	0.86	$F_{1,40}=5.71$	*0.02*	$F_{1,40}=36.14$	*<0.0001*	$F_{1,40}=1.02$	0.32
Cocc×Carot	$F_{1,40}=1.68$	0.20	$F_{1,40}=0.04$	0.84	$F_{1,40}=0.002$	0.96	$F_{1,40}=0.16$	0.69
within subjects								
time	$F_{4,37}=1.43$	0.24	$F_{2,39}=13.44$	*<0.0001*	$F_{2,39}=69.65$	*<0.0001*	$F_{2,39}=7.12$	0.11
time×Cocc	$F_{4,37}=2.94$	*0.03*	$F_{2,39}=7.05$	*0.002*	$F_{2,39}=4.27$	*0.02*	$F_{2,39}=0.07$	0.93
time×Carot	$F_{4,37}=2.84$	*0.03*	$F_{2,39}=9.32$	*0.0005*	$F_{2,39}=162.70$	*<0.0001*	$F_{2,39}=1.48$	0.24
time×Carot×Cocc	$F_{4,37}=4.10$	*0.007*	$F_{2,39}=0.48$	0.62	$F_{2,39}=0.78$	0.46	$F_{2,39}=1.12$	0.34

3. RESULTS

(a) *Parasite load*

Before the start of the experiment, the level of infection was very low and did not differ between groups (ANOVA, $F_{3,43}=0.79$, $p=0.51$). The number of oocysts shed by uninfected males remained very low during the experiment irrespective of the carotenoid treatment. By contrast, males experimentally infected with *Isospora* showed an increase in the number of oocysts shed (figure 1; table 1a). The significant interaction between time, carotenoid and coccidia (table 1a) suggests that there are different temporal dynamics of oocyst shedding between the two infected groups. Indeed, the peak of oocyst production occurred two weeks after the inoculation in C+ males, and only one week after the inoculation in C− males (figure 1). However, the number of oocysts shed by the two infected groups was not significantly different (table 1a).

(b) *Change in bill colour*

Male bill colour did not differ between groups at the start of the experiment (ANOVA, $F_{3,43}=0.66$, $p=0.72$). Both infection and carotenoid availability influenced bill colour changes over the time course of the experiment (table 1b). Two weeks after the start of the experiment, C− infected males exhibited paler bills, whereas no change was observed in the other groups. At the end of the experiment, C+ uninfected males had significantly redder bills while the bill colour of C+ infected males remained unchanged, as in the control group. Between the day 14 and the end of the experiment, C− males infected with coccidia showed no change in bill colour (figure 2a).

(c) *Change in plasma carotenoid concentration*

Initial plasma concentration of carotenoids did not differ between the four groups (ANOVA, $F_{3,43}=0.86$, $p=0.61$). During the experiment, carotenoids varied across time, depending on infection and carotenoid treatments (table 1c). Within two weeks of inoculation, C+ males showed a significant increase of carotenoid concentration in their plasma. However, the increase was higher in non-infected birds than in infected ones. On the other hand, C− males showed no significant change in plasma carotenoids irrespective of their infection status (figure 2b). The same pattern was observed on day 28;
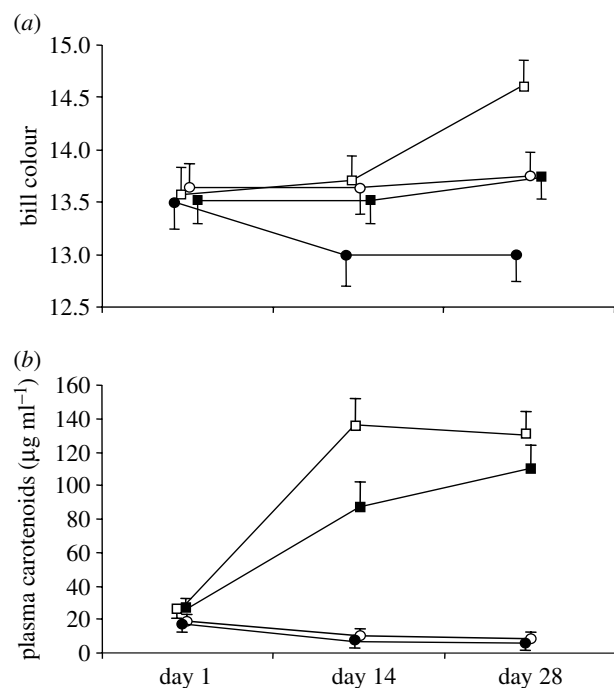
Figure 2. Change in (a) bill colour and (b) plasma carotenoids over the experiment period depending on the availability of carotenoids and the infection status of birds (mean±s.e.). Open squares, carotenoid supplemented and uninfected; filled squares, carotenoid supplemented and infected; filled circles, non-carotenoid supplemented and infected; open circles, non-carotenoid supplemented and uninfected.

only the two supplemented groups had more carotenoids than at the start of the experiment. Uninfected males showed the same concentrations as they had on day 14, whereas infected males circulated more carotenoids than on day 14. As a result, carotenoids reached the same concentration for both groups, but it took more time for infected males to reach this concentration (figure 2b). Plasma carotenoids were not significantly affected by coccidian infection at day 28 in C− males (figure 2b).

(d) *Change in body mass*

Mass of male blackbirds did not differ between groups before the start of the experiment (ANOVA, $F_{3,43}=0.97$, $p=0.42$). None of our treatments affected body mass of blackbirds (table 1d).

4. DISCUSSION

Despite extensive correlation evidence of the effect of infection and carotenoid availability on carotenoid allocation to sexual display, our study is the first to support this relationship experimentally. We found that (i) male blackbirds provided with an extra dose of carotenoids circulated more carotenoids in blood and developed more brightly coloured bills, (ii) carotenoid availability did not prevent parasite multiplication but slowed down their replication rates, (iii) the presence of coccidia slowed down the assimilation of carotenoids in the blood for carotenoid-supplemented birds, and (iv) infestation by *Isospora* was rapidly mirrored by the blackbirds' bill colour when carotenoid availability was limited.

The importance of carotenoid availability in the coloration of secondary sexual characters is now largely supported (Kodric-Brown 1989; Blount *et al.* 2003; Alonso-Alvarez *et al.* 2004; McGraw & Ardia 2004). Our results were consistent with these previous findings, as male blackbirds with access to a food source enriched in carotenoids (lutein and zeaxanthin) had higher concentrations of plasma carotenoids, which resulted in the development of more brightly coloured bills. Therefore, although little is known on the amount of carotenoids that blackbirds include in their natural diet and on variation of this amount, we might expect that the intensity of bill colour may reveal the foraging success of male blackbirds in the wild. However, within uninfected males, the concentration of plasma carotenoids did not increase after 14 days of dietary supplementation and tended to stabilize at approximately 100 $\mu g \ ml^{-1}$ despite constant supplementation. This suggests that the 'carrying capacity' (i.e. the maximum carotenoid load that could be absorbed or transported) was reached and/or that the amount of circulating carotenoids might be maintained at a stable level preventing increase above a certain optimum due to a physiological regulation, for instance by diffusion to the storage organs (Surai & Speake 1998; Surai 2002; Biard *et al.* 2006).

Many studies have confirmed the immunostimulant properties of carotenoids (Chew 1993; Van Poppel *et al.* 1993; McGraw & Ardia 2003; Grether *et al.* 2004). However, few works have addressed directly whether carotenoid availability enhances parasitic resistance. There is some indirect support of a positive relationship between the quantity of carotenoids available for an individual and its capacity to resist parasites (Grether *et al.* 2004; Hill & Farmer 2005; Kolluru *et al.* 2006). In this study, we used lutein and zeaxanthin as carotenoid pigments. The immunostimulant properties of these two pigments remain controversial: some studies have provided evidence for their immunoenhancing effects (Kim *et al.* 2000; Blount *et al.* 2003; McGraw & Ardia 2003), whereas other studies did not find such evidence (Navara & Hill 2003; Fitze *et al.* 2007). Here, carotenoid availability did not prevent parasites multiplications (i.e. infected males shed the same quantity of oocysts over the course of the experiments whatever the carotenoid treatment) but rather slowed down their replication rate (i.e. peak of infection appears one week later in C+ group compared with the C− group). Further experiments are therefore required to elucidate whether a delayed multiplication of the parasite might be beneficial for the host. However, our results suggest that lutein and zeaxanthin

have immunoenhancing properties in blackbirds as already observed in preliminary experiments (J. Moreau 2007, unpublished results). On the other hand, Navara & Hill (2003) failed to detect difference in disease dynamic between male American goldfinches provided with a carotenoid-rich food and control males, suggesting that males' ability to fend of a pathogen is not carotenoid limited or that these two pigments do not promote immune function in this species. This last conclusion was also proposed by Fitze *et al.* (2007) in a study on great tits (*Parus major*). Clearly, further studies are needed to determine the effects of these two pigments on immunity in natural populations.

Given that lutein and zeaxanthin are involved in bill coloration and showed immunoenhancing effects in our study, they can be traded off between ornamentation and parasitic resistance. Infestation by *Isospora* was rapidly mirrored in blackbirds' bill colour that was depressed quickly (within two weeks) in C− infected males. Conversely, bills of C+ infected birds did not change over the course of the experiment, suggesting that males with a high carotenoid availability might be able to cope with the costs associated with parasitic infestations and thus maintain their bill coloration. These findings support the idea that sexual displays may signal male infection status as already observed in some biological models (e.g. Hill & Brawner 1998; Brawner *et al.* 2000; Horak *et al.* 2004; Martinez-Padilla *et al.* 2007). Previous studies, including in blackbirds, that used antigenic challenges provided direct support for the idea that immune defences and sexual signals compete for carotenoids (Blount *et al.* 2003; Faivre *et al.* 2003). Our results extend this finding, suggesting that carotenoid supplementation allows individuals to cope with all the costs induced by parasites, and not only the cost associated with immune response.

While our experiment was not designed to assess the physiological mechanisms that link coccidian infection, circulating carotenoids and bill colour, it still allowed us to examine whether different carotenoids treatments may influence the outcomes of the infection. Coccidian infestations are known to produce a large amount of free radicals (Allen 1997*a*,*b*) and are highly immunogenic (Martin *et al.* 1997; Yun *et al.* 2000; Smith *et al.* 2002; Horak *et al.* 2004). Infected birds were therefore forced to fight off the infection. Carotenoids may then have been allocated to antioxidant defences against free radicals (Bendich 1989), as well as to immune responses, by enhancing the abilities of macrophages, killer T cells and cytotoxic T cells (Bendich 1989; Chew 1993; Lozano 1994, 2001). Previous experimental infections of chickens by coccidia resulted in a decreased concentration of carotenoids in peripheral blood as well as in visible tissues (*e.g.* Yvoré & Mainguy 1972; Ruff *et al.* 1974), which is consistent with the process of carotenoid recruitment from storage tissues to sustain immune and antioxidant functions. In our study, carotenoids may have been invested in these self-maintenance functions at the expense of bill colour.

Our results contrast with those of Fitze *et al.* (2007) who did not find evidence that lutein and zeaxanthin are traded off between ornamentation and immune function. They suggested that (i) these two carotenoids are not crucial for immune functions and (ii) because immune activation is energy demanding and consumes lipids,

carotenoid absorption and/or transportation may be reduced through a lowered availability of the lipids required for these two functions (see also Surai 2002; Tschirren *et al.* 2003; McGraw *et al.* 2005). The honesty of carotenoid-based signals could then be maintained by a physiological limitation on the absorption and/or transport of pigments. However, if absorption and/or transportation of carotenoids had been limited in our case study, we would have expected a decrease of circulating carotenoids independently of their availability. Instead, C+ infected males showed a clear increase of plasma carotenoid. Therefore, our results do not support the absorption/transportation hypothesis.

Based on the assumption that parasitic resistance consume carotenoids, we would have expected a reduction of circulating carotenoids in infected birds compared with uninfected ones. However, we found that the patterns of circulating carotenoids were similar between C− infected and C− uninfected birds. Two non-exclusive hypotheses may be proposed to explain this result. First, infected birds may have withdrawn carotenoids from storage tissues to transport and provide the immunostimulant and antioxidant resources required to fight infection. Second, plasma carotenoids usually invested (often as precursors) in bill colour may have been retained in the plasma to remain available for fighting infection. Such a mechanism is consistent with the observed decrease of C− infected male bill colour in this study, and with previous studies on male blackbirds (Faivre *et al.* 2003). These two hypotheses raise the question of the biological significance of circulating carotenoids in relation to sexual signalling and the reaction to infection. It may also be possible that we failed to accurately detect the dynamic of circulating carotenoids (i.e. the balance between accumulation of carotenoids from the diet, their use and their release from storage tissues) over the course of the experiment, because the time elapsed between the two assessments (two weeks) was not relevant.

Within the two supplemented groups, we observed a clear difference in the timing of carotenoid circulation between uninfected and infected birds. The fact that it took more time for infected males to reach a high concentration has several possible explanations. First, coccidia are parasites of the gastrointestinal tract, and it is known that pathogenesis of *Eimeria* spp. (a well-studied coccidia genus) disturbs permeability of epithelial cells and affects absorption of nutriments, including carotenoids (Yvoré & Mainguy 1972; Ruff & Fuller 1975; Allen 1997*a*,*b*; Hoste 2001). Accordingly, a reduction of body mass in infected birds could be expected, as been shown in young turkeys (*Meleagris gallopavo*; Augustine & Thomas 1981). However, this seems unlikely, or at least such a mechanism would have been reduced in our study, because (i) infected birds did not reveal any significant body mass deviation and (ii) circulating carotenoids increased in plasma of infected males, suggesting that absorption continued. In addition, previous studies failed to detect any change in body mass after infection with *Isospora* sp. (Mazgajski & Kedra 1998; Kruszewicz & Dyrcz 2000), suggesting that this coccidian genus does not cause strong damage to the epithelial cells of the gut. Secondly, we might hypothesize that plasma carotenoids were used to fight off the infection. Indeed, the intensity of infection peaked two weeks after the start of the experiment. This could explain why circulating carotenoids increased less in C+ infected males than in C+ uninfected males over the first two weeks of the experiment, and why circulating carotenoids still increased in the C+ infected group during the two last weeks.

Overall, our results suggest that bill colour of blackbirds is an honest signal of male infection status. However, when males are provided with a carotenoid-rich diet they can maintain the expression of costly carotenoid-dependent signals while paying the cost of being infected (Lozano 2001). In addition, our manipulation of both carotenoid availability and infection status at the same time clearly suggests a trade-off in carotenoid allocation between parasitic resistance to this intestinal parasite and secondary sexual traits. However, the physiological mechanisms that govern the honesty of the signal remain unclear. Further studies are required to investigate whether carotenoid availability modulates parasite multiplication dynamics in the host, and whether parasites influence carotenoid circulation, storage and allocation to these signals.

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