

Carotenoid-based bill colour is an integrative signal of multiple parasite infection in blackbird

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Abstract In the study of parasite-mediated sexual selection, there has been controversial evidence for the prediction that brighter males should have fewer parasites. Most of these studies have focused on one parasite species. Our aim was to investigate the expression of carotenoid-based coloured signals in relation to patterns of multiple parasite infections, to determine whether colour reflects parasite load of all parasite species, or whether different relationships might be found when looking at each parasite species independently. We investigated the relationship between bill colour, body mass and plasma carotenoids and parasite load (feather chewing lice, blood parasite *Plasmodium* sp., intestinal parasites cestodes and coccidia) in the blackbird (*Turdus merula*). Bill colour on its own appeared to be a poor predictor of parasite load when investigating its relationships with individual parasite species. Variation in parasite intensities at the community level was summarised using principal component analysis to derive synthetic indexes of relative parasite species abundance and absolute parasite load. The relative abundance of parasite species was strongly related to bill colour, plasma carotenoid levels and body mass: birds with relatively more cestodes and

chewing lice and relatively less *Plasmodium* and coccidia had a more colourful bill, circulated more carotenoids and were heavier. These results suggest that bill colour more accurately reflects the relative intensities of parasite infection, rather than one-by-one relationships with parasites or absolute parasite burden. Investigating patterns of multiple parasite infection would thus improve our understanding of the information conveyed by coloured signals on parasite load.

Keywords Avian malaria · Carotenoid-based signals · Host-parasite interactions · Phthiraptera · Parasite-mediated sexual selection

Introduction

The Hamilton and Zuk hypothesis of parasite-mediated sexual selection proposes that bright ornaments have evolved to signal parasite resistance to potential mates (Hamilton and Zuk 1982). Within this context, carotenoid-based signals have been particularly studied because limited availability of carotenoid pigments should lead to a trade-off in their allocation to immunity and colour display that would ensure honesty in these signals (Lozano 1994; Møller et al. 2000; Shykoff and Widmer 1996). There has been controversial evidence for the prediction that brighter males should have fewer parasites. This may result from variable negative effects of the parasites under study, variable trade-offs and allocation strategies for carotenoids among hosts and among host-parasite systems, and/or the importance to consider parasite resistance rather than parasite load (reviews and discussions in Hill 2006; Møller et al. 2000; Møller et al. 1999; Shykoff and Widmer 1996). Another explanation for the variety of relationships

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found between parasite load and expression of bright signals might be linked to the fact that, because of the difficulty in taking into account the whole parasite community, the majority of studies have focused on single parasite or group of parasite species (Dawson and Bortolotti 2006; Hõrak et al. 2001). Indeed, few have investigated the expression of carotenoid-based signals in relation to patterns of multiple parasite infections (Figuerola et al. 2005; Hatchwell et al. 2001). Individuals harbour a community of parasites and endure multiple infections, with parasite species often co-occurring and interacting within hosts, leading to correlations between different parasite species prevalence or intensity (e.g. Cox 2001; Holmstad et al. 2008; Holmstad and Skorping 1998). For example, recent studies have shown that the intensities of two parasite species could be positively (Holmstad et al. 2008; Holmstad and Skorping 1998) or negatively (Goüy de Belloq et al. 2007; Hatchwell et al. 2000) correlated within host. Therefore, investigating the relationship between brightness of sexual signals and parasite load focusing on single, or a subset of parasite species might lead to inaccurate interpretations derived from partial patterns (Holmstad et al. 2008). In this context, different relationships between colour and parasite load might be found depending on the parasite species considered and the underlying relationships among parasite species and their relative impact on host fitness.

Most studies of parasite-mediated sexual selection in birds have focused on plumage colour (Hill and McGraw 2006). However carotenoid-based skin or epithelium colours relying on constant pigment deposition are more dynamic traits susceptible to convey accurate information about an individual's current body condition and health status, in contrast with plumage colour, which is determined at moult. Moreover, carotenoid-based colours of skin and more keratinized tissues have been found to reflect current status at different time scales (Pérez-Rodríguez et al. 2008; Pérez-Rodríguez and Viñuela 2008). Carotenoid-based bill colour has been shown to be reduced by immune responses induced by controlled immune challenges (in the blackbird *Turdus Merula*, (Faivre et al. 2003a); in the zebra finch *Taeniopygia guttata*, (Alonso-Alvarez et al. 2004; Gautier et al. 2008); in the mallard *Anas platyrhynchos*, (Peters et al. 2004)) and by endoparasites (coccidia and nematodes; in the blackbird: (Baeta et al. 2008); in red-legged partridge *Alectoris rufa*, (Mougeot et al. 2009)). This decrease in bill colour likely reflects variation in plasma carotenoids levels (in the zebra finch, (Blount et al. 2003b; McGraw and Ardia 2003); in the mallard, (Peters et al. 2004)). Indeed immune activation with various antigens eliciting inflammatory and/or humoral immune response has been reported to quickly reduce circulating carotenoid levels

((Alonso-Alvarez et al. 2004; McGraw and Ardia 2003; Peters et al. 2004); in the blackbird: (Biard et al. 2009); in the domestic chicken *Gallus g. Domesticus*, (Koutsos et al. 2003)) but see (in the Eurasian kestrel *Falco tinnunculus*, (Costantini and Dell'Omo 2006); in the greenfinch *Carduelis chloris*, (Hõrak et al. 2006)). Endoparasites also interfere with carotenoid absorption in the intestine and infection decreases plasma carotenoid levels (e.g. Allen 1992; Hõrak et al. 2004a). However, a positive relationship between carotenoid-based skin colour and carotenoid stores and other endoparasites (cestodes) has also been reported (Figuerola et al. 2005). This suggests that the relationship between carotenoid-based skin or bill colours and parasites might be complex and depend on the parasite species or the host-parasite system investigated.

In this study, we investigated the relationships between expression of colour and parasite load, both considering individual parasite species and taking patterns of multiple infections into account. Our aim was to determine whether colour uniformly reflects parasite load of each parasite species, in which case qualitatively similar positive, negative or null relationships should be found with all parasites, or whether different relationships might be found when looking at each parasite species independently. The latter would imply that a valid assessment of what information coloured signals convey on parasite load would only be obtained when investigating multiple parasite loads. Such a correlative study is not designed to infer causation in the relationships among the variables considered, but to describe patterns while taking into account the complexity of host parasite systems. This should help in the interpretation of further (correlative or experimental) work. We used the common blackbird, *T. merula* as a model species. Bill colour in this species is likely to be a sexually selected trait (Bright and Waas 2002; Faivre et al. 2001) and has been shown to reflect immune system activation and responsiveness (Faivre et al. 2003a,b) and to be positively correlated to plasma carotenoids (Biard et al. 2009). In two different studies, bill colour also reflected coccidial (Baeta et al. 2008) but not malarial infection (Hatchwell et al. 2001) in males. In a first attempt to take the effects of multiple infections into account, we captured male blackbirds and assessed ectoparasites and endoparasites (blood parasites and intestinal parasites) load, bill colour, as well as body mass and plasma carotenoids, to test the hypothesis that bill colour would reflect parasite load. Although the number of different parasites identified here likely represents a subset of the whole parasite community, this includes each of the most studied parasites types in the context of the evolution of sexual signals, allowing a relevant test of the relationship between patterns of multiple infections and the expression of a coloured signal.

Materials and methods

Trapping, general maintenance and general method

Twenty-two adult male blackbirds (2 years old and older) were caught using mist-nets in urban parks of Dijon (France, 47°19'N, 5°02'E) in February 2007. On the day of capture (day 0), birds were isolated in individual outdoor cages (69.5×44.5×82.5 cm) and fed ad libitum with food for large Turdid species (COFNA) and tap water. Water and food were renewed daily. After 1 week of acclimatisation (day 7), we assessed: (1) ectoparasite and intestinal parasite loads, (2) bill colour and (3) body mass and tarsus length. Body mass was recorded to the nearest 0.1 g using an electronic balance (Scout Pro). Right and left tarsus lengths were measured with a calliper (precision 0.02 mm). A blood sample was taken from the brachial vein using sterile needles and heparinised micro-capillary tubes, to measure plasma carotenoid concentration and count blood parasites. We repeated these measures 1 week later (day 14). One additional measure was performed on day 10 for intestinal parasites to account for the strong daily variability in the release of eggs of these parasites (Filipiak et al. 2009; Hōrak et al. 2004a). An acclimatisation period was necessary to let the birds recover from the stress of capture and captivity which might take longer when temperature is low and birds face energetic costs of thermoregulation. We therefore avoided further adding the stress of manipulation during measurements (approximately 20 min per bird), as an important stress at the time of the first measures might have induced a change in the birds' resistance and parasite loads at the subsequent measures. We cannot rule out the possibility that good nutritional conditions might have modified plasma carotenoids levels, bill colour, body mass, and parasite infections already during the first week of captivity. However, there was no significant change in plasma carotenoid levels, body mass, bill colour or parasite loads among measurement sessions (paired samples *t* tests and paired samples Wilcoxon's signed-rank tests; all *p* values > 0.05). This suggests that even if a slight change might have occurred in the first days of captivity, there was no evidence of an amelioration of the birds' health over the course of the study due to unlimited access to food. Moreover, in the blackbird, a previous study showed that decrease in bill colour following immune activation occurred within 14 days, while an increase in bill colour occurred only after 28 days of carotenoid-supplemented diet (Baeta et al. 2008). This seems to indicate that diet composition and/or abundance may influence bill colour, but on a longer time scale than our 1 week acclimatization period. For all variables, the mean of the two or three measures was used in subsequent analyses since the repeatability of the measures was always high and

significant (see below). At the end of the survey, on day 15, birds were released in large outdoor aviaries (220×150×250 cm).

Assessment of parasitic load

Intestinal parasites An aluminium sheet was placed on the bottom of cages for 24 h to collect faeces. Faecal samples were weighed at the nearest ± 0.01 g with an electronic balance (ScoutPro) and placed in a 50 mL Falcon tube. Samples were gently homogenized with 14 mL of a Sheeter solution (solution of sugar 45%). Parasites were counted in a McMaster chamber in which 600 μL of the sample solution was placed and left for 10 min to allow the oocysts and eggs to float. We focused our count on two different intestinal parasites: coccidians and cestodes, since very few nematodes were found: in two individuals one nematode species could not be identified, in only three individuals one egg of a *Capillaria* sp. (Capillariidae) was found, and very few *Trichostrongylus* sp. (Trichostrongylidae) were also found. Two counts were conducted for each faecal sample and the mean number of oocysts was used (repeatability of parasite counts per field and per individual are given in Filipiak et al. 2009). Coccidian and cestode concentrations were expressed as the mean number of parasites per gram of faecal sample over the three sampling days (days 7, 10 and 14). The repeatability of the measure was high and significant (intra-class correlation coefficient: $r=0.83$, $n=22$, $p<0.0001$, Lessells and Boag 1987)

Ectoparasites Each bird was carefully inspected during 5 min over the whole body surface to detect all visible ticks and chewing lice (Clayton and Walther 1997; Grégoire et al. 2002). Ticks are mainly located on the head in this species (Grégoire et al. 2002), and urban blackbirds have very low infestation rates by ticks (2% of birds Grégoire et al. 2002), thus a particular attention was given to this body area in order to limit the risk of missing ticks. Chewing lice were counted by carefully examining the bird's head, throat, belly and rump. Ectoparasites were not removed during inspection. The repeatability of the measures between days 7 and 14 was high and significant (intra-class correlation coefficient: $r=0.85$, $n=22$, $p<0.0001$, Lessells and Boag 1987).

Blood parasites Thin blood smears were made for each bird immediately after blood sampling. Slides were air-dried, then immediately fixed in absolute methanol for 5 min and stained with a Giemsa solution for 40 min. Blood smears were examined under the microscope (x 1000 magnification, oil immersion). The number of parasitized cells in each smear was determined on the basis of the examination of 10,000 erythrocytes (Hatchwell et al. 2000).

Blood parasites were identified using the description of Valkiunias (2005). As it is difficult to identify *Plasmodium* at the species level from blood stage alone we referred only to the genus in this study (Bennett et al. 1993). Other parasites such as *Haemoproteus*, *Leucocytozoon* or *Trypanosoma* were found only scarcely (results not shown here) and these species were thus excluded from the analysis. This is consistent with previous studies in this urban blackbird population reporting that the most frequent haematozoan parasite is *Plasmodium* (Barroca 2005). Although haematozoan parasite counts from blood smears were shown to underestimate the actual parasite prevalence, they nevertheless significantly and positively correlated with parasitaemia (Bentz et al. 2006). In the context of our study, haematozoan parasite counts therefore provided a biologically meaningful measure of infection. The repeatability of the measure was high and significant (intra-class correlation coefficient: $r=0.96$, $n=22$, $p<0.0001$, Lessells and Boag 1987).

Bill colour scoring

Colour was assessed 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) following a method used previously in this species (Baeta et al. 2008; Faivre et al. 2001). This scale is composed by 0.5 steps. This colouration index has been shown to be highly correlated to measures of bill colour obtained with a spectroradiometer (Faivre et al. 2001). Observed bill colour scores ranged from index 11 to 13.5. Scoring was always performed by the same observer under the same light conditions, and were highly repeatable (intra-class correlation coefficient: $r=0.88$, $n=22$, $p<0.0001$, Lessells and Boag 1987).

Plasma carotenoids concentration

After collection, blood samples were immediately centrifuged (4,000 r.p.m. at 4°C for 5 min) and plasma stored in Eppendorf tubes at -80°C until later analysis. Concentration was assessed using a standardised colorimetric technique (Alonso-Alvarez et al. 2004; Baeta et al. 2008). Twenty microlitres of plasma was diluted in 180 µL of absolute ethanol; gently vortexed and flocculent proteins were precipitated by centrifugation at 1,500×g for 10 min. Optical density of the supernatant was determined with a spectrophotometer at 450 nm. Carotenoid concentration was determined from a standard curve of lutein. The repeatability of the measure was high and significant (intra-class correlation coefficient: $r=0.86$, $n=22$, $p<0.0001$, Lessells and Boag 1987).

Statistical analysis

Statistical tests were performed using JMP software (Version 3.2.2, SAS Institute Inc.). A matrix of Kendall's τ correlations was created to investigate the associations between different species of parasites, since intensities of most parasite species were not normally distributed (Holmstad and Skorping 1998). Principal component analysis on log-transformed and standardised data was used to summarize the information contained in the different parasite abundances into two orthogonal (uncorrelated) principal components describing an individual's overall parasite load. Tarsus length and body mass were not significantly correlated ($r_{\text{Pearson}}=0.08$, $p=0.73$), and running the analyses with an index of body condition instead of body mass did not change any of the results presented in the manuscript. Only the results of the analyses with body mass are thus presented. Potential relationships between morphological characters (bill colour, carotenoid content and body mass) and parasite intensity for each parasite species, and that with PC1 and PC2 were explored with Spearman's rank correlation coefficients. Exact p values are reported, and Bonferroni corrections were not applied (Moran 2003; Nakagawa 2004).

Results

Parasite prevalence and relationship among parasite intensities

Only one type of ectoparasite was found in blackbirds, the feather-chewing lice *Philopterus* sp. (Phthiraptera: *Philopteridae*) that occurred in half of the birds at low intensity. Birds did not have any ticks. All birds were infected with one species of cestode *Hymenolepis* sp. and oocysts of one species of coccidian: *Isospora turdi*. Eighty percent of blackbirds were infected by *Plasmodium* sp. Prevalence and intensity of the different parasite species are reported in Table 1.

By looking at all possible correlations between the intensities of different parasite species, we found two positive associations. Firstly, chewing lice intensity was significantly positively correlated with cestode intensity. Secondly, *Plasmodium* intensity was significantly positively correlated to coccidia intensity (Table 2). There was also a marginal negative correlation between chewing lice intensity and *Plasmodium*.

Principal component analysis on parasite loads

PC1 accounted for 57.85% of the variation in parasite load with positive loading coefficients for *Plasmodium* and coccidia and negative loading coefficients for chewing lice

Table 1 Prevalence (percentage of hosts infected), mean intensities (mean number of parasites per infected host)±SE, and range of intensity of different parasite species recorded in blackbirds (n=22)

	Prevalence	Mean intensity	Range
Chewing lice <i>Philopterus</i> sp.	45.45	7.61±2.60 ^a	0–30 ^a
<i>Plasmodium</i> sp.	81.81	12.55±3.41 ^b	0–50 ^b
Cestode <i>Hymenolepis</i> sp.	100	390.15±85.5 ^c	25–1,262 ^c
Coccidia <i>Isospora turdi</i>	100	7,766.25±1,716 ^d	538–26,449 ^d

^a Number of chewing lice found during 5 min inspection of the bird’s feathers

^b Number of parasitized cells per 10,000 erythrocytes

^c Number of cestode eggs per gram of faecal sample

^d Number of *Isospora* coccidia oocysts per gram of faecal sample

and cestodes (Table 3). Therefore, PC1 reflected a clear opposition between two groups of parasites: chewing lice and cestodes on the one hand, and *Plasmodium* and coccidia on the other hand. A score for each individual blackbird along PC1 was thus used as an index of parasitism describing the relative abundance of different parasite species harboured by their host: Individuals with low PC1 values have relatively more cestodes and chewing lice, and fewer *Plasmodium* and coccidia, and vice versa. PC2 explained 25.17% of the variation with positive loadings for all parasite species intensities. PC2 thus reflects the absolute parasite burden: an individual with high PC2 score carried many parasites of all four species, and vice versa.

Relationships between bill colour, body mass plasma carotenoids and parasite loads

Bill colour was significantly positively correlated with body mass and plasma carotenoids (spearman rank correlation coefficients: $r=0.56, p=0.006$ and $r=0.46, p=0.02$, respectively). Body mass and plasma carotenoid concentration were also significantly positively correlated (spearman rank correlation coefficient: $r=0.54, p=0.009$). Body mass was not related to parasite load for any parasite species (Table 4). Bill colour was significantly positively related to the intensity of parasitic infestation by chewing lice, but not to the other parasite species (Table 4). Plasma carotenoid

concentration was significantly negatively related to *Plasmodium* and coccidia intensities, and positively to cestodes and chewing lice intensities (Table 4).

There was a significant negative correlation between PC1 and (1) bill colour, (2) plasma carotenoid concentration and (3) mass (Fig. 1). Blackbirds harbouring many cestodes and chewing lice and few *Plasmodium* and coccidia were heavier, had a more colourful (more orange) bill, and circulated more carotenoids. Conversely, blackbirds with many *Plasmodium* and coccidia, but few cestodes and chewing lice, were lighter, had a less colourful (more yellow) bill and circulated less plasma carotenoids (Fig. 1). On the other hand, PC2 was not significantly correlated to bill colour, body mass or plasma carotenoids (Spearman’s rank correlation coefficients: $r=0.39, p=0.08$; $r=0.04, p=0.87$ and $r=0.29, p=0.20$, respectively).

Discussion

Our aim in this study was to investigate the relationship between bill colour, body mass and parasite load, taking

Table 3 Principal components analysis of the intensities of four parasite species: one ectoparasite chewing lice, one malarial blood parasite (*Plasmodium*), and two intestinal parasites (cestode and *Isospora* coccidia) in blackbirds

Parameter	PC1	PC2
Eigenvalue	2.31	1.07
Explained variance (%)	57.85	25.17
Component loading		
Chewing lice	-0.49	0.49
<i>Plasmodium</i>	0.53	0.45
Cestode	-0.48	0.54
Coccidia	0.50	0.52

Component loadings describe the relationships between the first two principal components (PC) and the variables from which they were derived

Table 2 Kendall’s τ rank correlations coefficients between the intensities of a chewing lice ectoparasite (Phthiraptera), one malarial blood parasite (*Plasmodium*), and two intestinal parasites (cestode and *Isospora* coccidia) in blackbird

Kendall’s τ, P	Chewing lice	<i>Plasmodium</i>	Cestode
<i>Plasmodium</i>	-0.28, 0.09		
Cestode	0.37, 0.03	-0.18, 0.24	
Coccidia	-0.21, 0.20	0.39, 0.01	-0.11, 0.48

Values in bold indicate a significant correlation

Table 4 Spearman's rank correlation coefficients between parasite intensities and bill colour, plasma carotenoid concentration, and body mass in blackbirds

	Bill colour		Plasma carotenoids		Mass	
	r_s	P	r_s	P	r_s	P
Chewing lice	0.50	0.01	0.49	0.02	0.39	0.07
<i>Plasmodium</i>	-0.24	0.28	-0.56	0.007	-0.29	0.19
Cestode	0.35	0.11	0.52	0.01	0.44	0.06
Coccidia	-0.21	0.35	-0.46	0.03	-0.11	0.60

Values in bold indicate a significant correlation

into account patterns of multiple parasite infection. The parasite community found in blackbirds was mainly composed of three endoparasite species and one type of ectoparasite, a feather chewing lice. Abundance of cestode and chewing lice were significantly positively correlated. Increased infestation with chewing lice has been proposed to result from a decrease in the energetically demanding preening behaviour, as a consequence of infection with cestodes (Holmstad et al. 2008). Intensities of infection

with coccidia and *Plasmodium* were also significantly positively correlated (see also Holmstad and Skorping 1998), which might reflect that it is difficult for an individual to control both infections at the same time (Cox 2001). These positive correlations among parasites may also reflect covariation between branches of the immune system, which may be differentially stimulated or inhibited by different parasites (e.g. Blount et al. 2003a; Roulin et al. 2007).

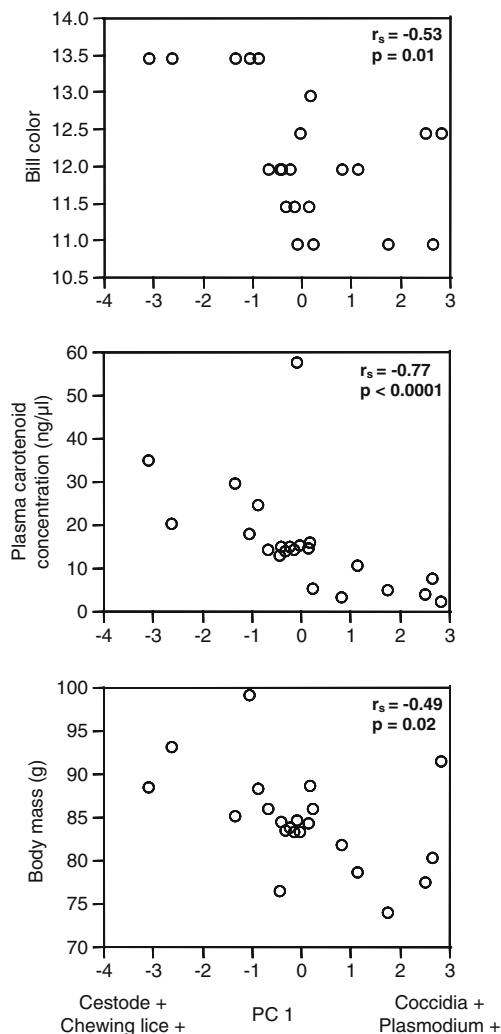


Fig. 1 Relationship between PC1 score summarizing relative ectoparasite and endoparasite load in blackbirds and (a) bill colour score, (b) plasma carotenoid concentration (ng/ μ l), and (c) body mass (g)

Relationships between bill colour, body mass and plasma carotenoids and parasite loads

Although bill colour, plasma carotenoids and body mass were significantly positively correlated to each other, their individual relationships with parasite intensities differed among them and with each parasite species considered. Body mass was not significantly correlated with parasite load for any parasite species, which might reflect the absence of significant negative effects, or that parasite load reflected chronic and controlled infections. Infection with coccidian parasites for example, are not always found to be associated with decreased host body mass (Kruszewicz and Dyrz 2000; Mazgajski and Kedra 1998) but see (Costa and Macedo 2005). In previous studies of the effects of parasitism on blackbirds, no relationship between body mass and *Plasmodium* was found in males (Hatchwell et al. 2001), and neither between body mass and infestation by feather mites of the genus *Proctohyllodes* (Bright et al. 2004). Results from this study showed that bill colour and plasma carotenoids were significantly positively related to the intensity of parasitic infestation by chewing lice. These relationships are on their own difficult to interpret, as it is not clear how an ectoparasite might positively influence carotenoid metabolism and deposition in ornaments. Bill colour did not reflect parasite load of any other parasite species. The absence of a significant relationship between bill colour and *Plasmodium* infection in males is consistent with findings reported in another blackbird population (Hatchwell et al. 2001). There was no significant correlation either between bill colour and coccidian parasites load. A decrease in carotenoid-based bill coloration is observed in controlled infection experiments with coccidia, as a result of a new and acute infection (Baeta et al. 2008 and

references therein). However, evidence for a negative relationship between the expression of a coloured signal and parasite load is less clear when chronic and potentially controlled infections are considered in correlative studies. Individuals able to control parasite infection may allocate proportionally more carotenoids in bill colour to maintain signal intensity, thereby cancelling any correlation between infection and the expression of the coloured signal. Plasma carotenoid concentration was significantly negatively related to *Plasmodium* and coccidia intensities. Such a negative relationship was expected with coccidia since this parasite directly interferes with nutrient absorption, especially that of carotenoids (Allen 1992; Hōrak et al. 2004a). Infection with coccidia is known to be highly immunogenic and associated to free radical production (Allen 1997a, b; Hōrak et al. 2004a; Yun et al. 2000), which might also induce a decrease in plasma carotenoid levels, through allocation of these pigments to immune function and/or antioxidant protection (Bendich 1993; Costantini 2008; Edge et al. 1997; Koutsos et al. 2003; Møller et al. 2000). However, to our knowledge, a similar negative relationship has not been reported yet for *Plasmodium* or other malarial parasites in birds, although decreased plasma antioxidant and carotenoid levels are well documented in humans with malaria (e.g. Das et al. 1996; Metzger et al. 2001; Nussenblatt et al. 2002). On the contrary, great tits *Parus major* infected with *Haemoproteus* were found to have higher plasma carotenoids and vitamin E levels than uninfected birds (Hōrak et al. 2004b). Plasma carotenoids were positively related to cestode intensity, which is consistent with positive relationships between cestode (but not nematode) load and carotenoid-based skin colour and carotenoid body stores reported in greylag geese *Anser anser* (Figuerola et al. 2005). Although plasma carotenoid levels were significantly related to all parasite species as well as bill colour, bill colour on its own appeared to be a poor predictor of parasite load when investigating its relationships with individual parasite species.

Synthetic indexes of parasite infection

When summarizing variation in parasite intensities at the community level and using principal component analysis to derive synthetic indexes of relative parasite species abundance (PC1) and absolute parasite load (PC2), clearer patterns emerged. The approach considering the parasite community to investigate its relationship with host phenotype is increasingly being used in immuno-ecology studies of immunocompetence (Gouÿ de Belloq et al. 2007; Vainikka et al. 2009). In addition, principal components analysis has been successfully used in studies of parasite communities to investigate the effects of parasites on secondary sexual traits (Masvaer et al. 2004) or body size

of the host (Ternengo et al. 2005). The relative abundance of parasite species was actually strongly related to bill colour, plasma carotenoid levels and body mass: birds with relatively more cestodes and chewing lice and relatively less *Plasmodium* and coccidia, had a more colourful bill, circulated more carotenoids and were heavier. On the other hand, the absolute parasite burden was not significantly related to variation in bill colour, plasma carotenoids and body mass. The descriptive nature of this study and the positive correlations between bill colour, plasma carotenoids and body mass do not allow us to infer causation in these patterns of covariation with indexes of parasitism. These results nevertheless suggest that bill colour more accurately reflects the relative intensities of parasite infection, rather than one-by-one relationships with parasites or absolute parasite burden. In addition, bill colour reflected plasma carotenoid levels and body mass. These results have two main implications.

Main implications

A main implication of this work is a caution on how the relationships between parasites, body mass and bright carotenoid-based signals should be interpreted, in the context of the study of parasite-mediated sexual selection using correlative data. Although our description of the parasite community of blackbirds was not exhaustive and this approach should be extended to include other parasite species less often studied (e.g. bacteria and viruses), our results suggest that it is important to consider multiple infections in a host, as well as the relationships among parasites at the community level, in order to investigate how parasites influence variation in body mass and coloured signal. Investigating the expression of coloured signal as a function of a single parasite, even when it is supposed or known to impose fitness costs on its host, might lead to inaccurate or partial conclusions. This might explain part of the discrepancies between studies that until now have in their majority focused on one parasite and reported contradictory results, with no significant relationship (as in our case with coccidia, cestode or *Plasmodium*), positive (as in our case with feather chewing lice) or negative relationships with carotenoid-based signals (reviewed in Hill 2006; Møller et al. 2000).

The second implication is on the information content of carotenoid-based coloured dynamic traits, such as bill and skin colours. Our results suggest that bill colour is an integrative signal of current status of multiple parasite infection and would thus convey information about different aspects of a male's quality to females. Females choosing a male with a brightly coloured beak avoid males highly infected with *Plasmodium* and coccidian, but they also mate with male parasitized with more feather lice and cestodes. It

will be interesting to investigate which parasites have lower or a higher deleterious effect on the female fitness in order to test if the female choice based on bill colour is adaptive. In addition, it could be beneficial for females to mate with brightly coloured males, which are heavier (see also Faivre et al. 2001) and have higher plasma carotenoid levels. This does not exclude the possibility that females could also use body mass (also correlated to PC1) as an indicator of parasite load, and rely on multiple signals for mate choice (Candolin 2003). However, it is beyond the aim of the manuscript to demonstrate whether females actually use bill colour alone or in combination with other morphological traits to choose a mate. Females might thereby be choosing males with better territory (Bright and Waas 2002; Bright et al. 2004; Faivre et al. 2001), and female body condition was interestingly found to be correlated to male bill colour (Faivre et al. 2001).

Conclusion

In the blackbird, we have shown that assessing the information conveyed by the expression of carotenoid-based bill colour on parasite load required investigating patterns of multiple parasite infections and taking into account the relationships among parasites at the community level. Should this be shown to be true also in other host-parasites system, this might change the way we investigate the proximate and ultimate sources of variation in the expression of colourful ornaments in relation to parasite-mediated sexual selection.

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References

- Allen PC (1992) Effect of coccidiosis on the distribution of dietary lutein in the chick. *Poultry Sci* 71(9):1457–1463
- Allen PC (1997a) Nitric oxide production during *Eimeria tenella* infections in chickens. *Poultry Sci* 76(6):810–813
- Allen PC (1997b) Production of free radical species during *Eimeria maxima* infections in chickens. *Poultry Sci* 76(6):814–821
- Alonso-Alvarez C, Bertrand S, Devevey GL, Gaillard M, Prost J, Faivre B, Sorci G (2004) An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. *Am Nat* 164(5):651–659
- Baeta R, Faivre B, Motreuil S, Gaillard M, Moreau J (2008) Carotenoid trade-off between parasitic resistance and sexual display: an experimental study in the blackbird (*Turdus merula*). *Proc R Soc B* 275:427–434
- Barroca M (2005) Hétérogénéité des relations parasites - oiseaux : importance écologique et rôle évolutif. Ph.D. Thesis, Université de Bourgogne
- Bendich A (1993) Physiological roles of antioxidants in the immune system. *J Dairy Sci* 76(9):2789–2794
- Bennett G, Bishop M, Peirce M (1993) Checklist of the avian species of *Plasmodium* Marchiafava & Celli, 1885 (Apicomplexa) and their distribution by avian family and Wallacean life zones. *Syst Parasitol* 26(3):171–179
- Bentz S, Rigaud T, Barroca M, Martin-Laurent F, Bru D, Moreau J, Faivre B (2006) Sensitive measure of prevalence and parasitaemia of haemosporidia from European blackbird (*Turdus merula*) populations: value of PCR-RFLP and quantitative PCR. *Parasitology* 133:685–692
- Biard C, Hardy C, Motreuil S, Moreau J (2009) Dynamics of PHA-induced immune response and plasma carotenoids in birds: should we have a closer look? *J Exp Biol* 212(9):1336–1343
- Blount JD, Houston DC, Møller AP, Wright J (2003a) Do individual branches of immune defence correlate? A comparative case study of scavenging and non-scavenging birds. *Oikos* 102:340–350
- Blount JD, Metcalfe NB, Birkhead TR, Surai PF (2003b) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* 300:125–127
- Bright A, Waas JR (2002) Effects of bill pigmentation and UV reflectance during territory establishment in blackbirds. *Anim Behav* 64:207–213
- Bright A, Waas JR, King CM, Cuming PD (2004) Bill colour and correlates of male quality in blackbirds: an analysis using canonical ordination. *Behav Processes* 65:123–132
- Candolin U (2003) The use of multiple cues in mate choice. *Biol Rev* 78:575–595
- Clayton DH, Walther BA (1997) Collection and quantification of arthropod parasites of birds. In: Clayton DH, Moore J (eds) *Host-parasite evolution: General principles and avian models*. Oxford University Press, Oxford, U.K., pp 419–440
- Costa FJ, Macedo RH (2005) Coccidian oocyst parasitism in the blue-black grassquit: influence on secondary sex ornaments and body condition. *Anim Behav* 70:1401–1409
- Costantini D (2008) Oxidative stress in ecology and evolution: lessons from avian studies. *Ecol Lett* 11:1238–1251
- Costantini D, Dell’Omo G (2006) Effects of T-cell-mediated immune response on avian oxidative stress. *Comp Biochem Physiol A* 145:137–142
- Cox FEG (2001) Concomitant infections, parasites and immune responses. *Parasitology* 122(Supplement S1):S23–S38
- Das BS, Thurnham DI, Das DB (1996) Plasma alpha-tocopherol, retinol, and carotenoids in children with falciparum malaria. *Am J Clin Nutr* 64(1):94–100
- Dawson RD, Bortolotti GR (2006) Carotenoid-dependent coloration of male American kestrels predicts ability to reduce parasitic infections. *Naturwissenschaften* 93:587–602
- Edge R, McGarvey DJ, Truscott TG (1997) The carotenoids as antioxidants—a review. *J Photochem Photobiol B* 41(3):189–200
- Faivre B, Pr eault M, Th ery M, Secondi J, Patris B, C ezilly F (2001) Breeding strategy and morphological characters in an urban population of blackbirds, *Turdus merula*. *Anim Behav* 61:969–974
- Faivre B, Gr egoire A, Pr eault M, C ezilly F, Sorci G (2003a) Immune activation rapidly mirrored in a secondary sexual trait. *Science* 300:103
- Faivre B, Pr eault M, Salvadori F, Th ery M, Gaillard M, C ezilly F (2003b) Bill colour and immunocompetence in the European blackbird. *Anim Behav* 65:1125–1131
- Figuerola J, Torres J, Garrido J, Green AJ, Negro JJ (2005) Do carotenoids and spleen size vary with helminth load in greylag geese? *Can J Zool* 83:389–395
- Filipiak L, Mathieu F, Moreau J (2009) Caution on the assessment of intestinal parasitic load in studying parasite-mediated sexual selection: the case of Blackbirds coccidiosis. *Int J Parasitol* 39(6):741–746

- Gautier P, Barroca M, Bertrand S, Eraud C, Gaillard M, Hammam M, Motreuil S, Sorci G, Faivre B (2008) The presence of females modulates the expression of a carotenoid-based sexual signal. *Behav Ecol Sociobiol* 62:1159–1166
- Goüy de Bellocq J, Ribas A, Casanova JC, Morand S (2007) Immunocompetence and helminth community of the white-toothed shrew, *Crocidura russula* from the Montseny Natural Park, Spain. *Eur J Wildl Res* 53:315–320
- Grégoire A, Faivre B, Heeb P, Cézilly F (2002) A comparison of infestation patterns by Ixodes ticks in urban and rural populations of the Common Blackbird *Turdus merula*. *Ibis* 144:640–645
- Hamilton WD, Zuk M (1982) Heritable true fitness and bright birds: a role for parasites? *Science* 218:384–387
- Hatchwell BJ, Wood MJ, Anwar M, Perrins CM (2000) The prevalence and ecology of the haematozoan parasites of European blackbirds *Turdus merula*. *Can J Zool* 78:684–687
- Hatchwell BJ, Wood MJ, Anwar MA, Chamberlain DE, Perrins CM (2001) The haematozoan parasites of Common Blackbirds *Turdus merula*: associations with host condition. *Ibis* 143(3): 420–426
- Hill GE (2006) Environmental regulation of ornamental coloration. In: Hill GE, McGraw KJ (eds) *Bird coloration 1 mechanisms and measurements*. Harvard University Press, Harvard, pp 507–560
- Hill GE, McGraw KJ (eds) (2006) *Bird coloration. 1. Mechanisms and measurements*. Harvard University Press, Harvard
- Holmstad PR, Skorping A (1998) Covariation of parasite intensities in willow ptarmigan, *Lagopus lagopus* L. *Can J Zool* 76:1581–1588
- Holmstad PR, Jensen KH, Skorping A (2008) Ectoparasite intensities are correlated with endoparasite infection loads in willow ptarmigan. *Oikos* 117:515–520
- Hörak P, Ots I, Vellau H, Spottiswoode C, Møller AP (2001) Carotenoid-based plumage coloration reflects hemoparasite infection and local survival in breeding great tits. *Oecologia* 126:166–173
- Hörak P, Saks L, Karu U, Ots I, Surai PF, McGraw KJ (2004a) How coccidian parasites affect health and appearance of greenfinches. *J Anim Ecol* 73:935–947
- Hörak P, Surai PF, Ots I, Møller AP (2004b) Fat soluble antioxidants in brood-rearing great tits: relations to health and appearance. *J Avian Biol* 35:63–70
- Hörak P, Zilmer M, Saks L, Ots I, Karu U, Zilmer K (2006) Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches. *J Exp Biol* 209:4329–4338
- Koutsos EA, Calvert CC, Klasing KC (2003) The effect of an acute phase response on tissue carotenoid levels of growing chickens (*Gallus gallus domesticus*). *Comp Biochem Physiol A* 135:635–646
- Kruszewicz A, Dyrz A (2000) Intestinal parasites in five bird species of the genus *Acrocephalus*. *Acta Ornithol* 35(2):153–158
- Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. *Auk* 104:116–121
- Lozano GA (1994) Carotenoids, parasites, and sexual selection. *Oikos* 70(2):309–311
- Masvaer M, Liljedal S, Folstad I (2004) Are secondary sex traits, parasites and immunity related to variation in primary sex traits in the Arctic charr? *Proc R Soc B* 271:S40–S42
- Mazgajski TD, Kedra AH (1998) Endoparasite *Isoospora* sp. (Coccidia, Eimeriidae) affects the growth of starling *Sturnus vulgaris* nestling. *Acta Parasitol* 43(4):214–216
- McGraw KJ, Ardia DR (2003) Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. *Am Nat* 162(6):704–712
- Metzger A, Mukasa G, Shankar AH, Ndeezi G, Melikian G, Semba RD (2001) Antioxidant status and acute malaria in children in Kampala, Uganda. *Am J Trop Med Hyg* 65(2):115–119
- Møller AP, Christe P, Lux E (1999) Parasitism, host immune function and sexual selection. *Q Rev Biol* 74(1):3–20
- Møller AP, Biard C, Blount JD, Houston DC, Ninni P, Saino N, Surai PF (2000) Carotenoid-dependant signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? *Avian Poult Biol Rev* 11(3):137–159
- Moran MD (2003) Arguments for rejecting the sequential Bonferroni in ecological studies. *Oikos* 100(2):403–405
- Mougeot F, Pérez-Rodríguez L, Sumozas N, Terraube J (2009) Parasites, condition, immune responsiveness and carotenoid-based ornamentation in male red-legged partridge *Alectoris rufa*. *J Avian Biol* 40:67–74
- Nakagawa S (2004) A farewell to Bonferroni: the problems of low statistical power and publication bias. *Behav Ecol* 15(6):1044–1045
- Nussenblatt V, Mukasa G, Metzger A, Ndeezi G, Eisinger W, Semba RD (2002) Relationship between carotenoids and anaemia during acute uncomplicated *Plasmodium falciparum* malaria in children. *J Health Popul Nutr* 20(3):205–214
- Pérez-Rodríguez L, Viñuela J (2008) Carotenoid-based bill and eye ring coloration as honest signals of condition: an experimental test in the red-legged partridge (*Alectoris rufa*). *Naturwissenschaften* 95:821–830
- Pérez-Rodríguez L, Mougeot F, Alonso-Alvarez C, Blas J, Viñuela J, Bortolotti GR (2008) Cell-mediated immune activation rapidly decreases plasma carotenoids but does not affect oxidative stress in red-legged partridges (*Alectoris rufa*). *J Exp Biol* 211:2155–2161
- Peters A, Delhey K, Denk AG, Kempenaers B (2004) Trade-offs between immune investment and sexual signalling in male mallards. *Am Nat* 164(1):51–59
- Roulin A, Christe P, Dijkstra C, Ducrest A-L, Jungi T (2007) Origin-related, environmental, sex, and age determinants of immunocompetence, susceptibility to ectoparasites, and disease symptoms in the barn owl. *Biol J Linn Soc* 90:703–718
- Shykoff JA, Widmer A (1996) Parasites and carotenoid-based signal intensity: how general should the relationship be? *Naturwissenschaften* 83(3):113–121
- Ternengo S, Levron C, Desideri F, Marchand B (2005) Parasite communities in european eels *Anguilla anguilla* (Pisces, Teleostei) from a corsican coastal pond. *Vie et Milieu* 55(1):1–6
- Vainikka A, Taskinen J, Löytynoja K, Jokinen EI, Kortet R (2009) Measured immunocompetence relates to the proportion of dead parasites in a wild roach population. *Funct Ecol* 23(1):187–195
- Valkiunias G (2005) *Avian malaria parasites and other Haemaphysporidae*. CRC, New York
- Yun CH, Lillehoj HS, Lillehoj EP (2000) Intestinal immune responses to coccidiosis. *Dev Comp Immunol* 24(2–3):303–324