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ORIGINAL ARTICLE

Is bill colouration in wild male Blackbirds (*Turdus merula*) related to biochemistry parameters and parasitism?

**Guillermo López · Ramón Soriguer ·
Jordi Figuerola**

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Abstract Studies of signalling and sexual selection in birds are increasingly focussing on the regulation of the expression of carotenoid-based ornaments. Brighter coloured ornaments are preferred during mate choice and are thought to signal resistance to parasites, immune capacity and health status. As the colouration of integuments is a dynamic trait, changes in colouration potentially reflect changes in individual condition. The bill of the male Common Blackbird (or Blackbird, *Turdus merula*) has become a model for studying the implications of carotenoid-coloured integuments in birds, and Blackbird bill colouration has been found to be related to reproductive ability and immune capacity. However, the relationship between bill colouration and parasites and health in this species remains unclear. We have analysed the association between bill colouration, parasites (blood and intestinal parasites) and health status indicators (standard haematological and plasma biochemistry variables) in free-ranging male Blackbirds during the breeding season. Bill colouration was found to be related to body condition, health status, stress and hydration and nutritional status, but the presence or load of the parasite groups studied was not found to be related to bill colouration. Moreover, parasites showed no clear aggregation patterns. Our results suggest that certain physiological constraints—rather than parasite infection—are the main cause of variability in the colouration of male Blackbird bills.

Keywords Carotenoids · Blackbird · Bill · Parasites · Birds · Colouration · Health · Biochemistry

Zusammenfassung Die Steuerung der Ausprägung karotenoidbasierter Ornamente ist ein zentrales Thema in Arbeiten zur Signalwirkung und sexuellen Selektion bei Vögeln. Prächtiger gefärbte Ornamente werden während der Partnerwahl bevorzugt. Es wird angenommen, dass sie (1) Parasitenresistenz, (2) Immunkompetenz und (3) Gesundheitszustand signalisieren können. Da die Körperoberfläche eine dynamische Färbung besitzt, können Veränderungen in der individuellen Kondition potentiell reflektiert werden. Der Schnabel von Amsel-Männchen wurde als Modell für diese Untersuchung zu den Effekten von karotenoidgefärbten Bestandteilen der Körperoberfläche bei Vögeln gewählt. Es ist bekannt, dass die Färbung der Amselschnäbel abhängig vom Reproduktionsvermögen und der Immunkompetenz ist. Gleichwohl ist der Zusammenhang mit Parasiten und Gesundheitsstatus unklar. Während der Brutsaison wurde das Verhältnis von Schnabelfärbung, Parasiten (Blut- und Darmparasiten) und Gesundheitsindikatoren (Haematokrit und biochemische Plasmawerte) von freilebenden Amsel-Männchen untersucht. Die Schnabelfärbung war abhängig von der Körperkondition, Gesundheitszustand, Stress, Flüssigkeitszufuhr und Ernährungszustand. Die Anwesenheit oder Belastung durch die untersuchten Parasitengruppen ergab keinen Zusammenhang mit der Schnabelfärbung. Darüber hinaus zeigten die Parasiten kein eindeutiges Ansammlungsmuster. Unsere Ergebnisse zeigen, dass eher bestimmte physiologische Belastungen

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G. López (✉) · R. Soriguer
Department of Biodiversity Conservation and Applied Ecology,
Estación Biológica de Doñana, Americo Vespucio s/n,
41092 Seville, Spain
e-mail: guiloza@gmail.com

J. Figuerola
Department of Wetland Ecology, Estación Biológica de Doñana,
Americo Vespucio s/n, 41092 Seville, Spain

als eine Parasiteninfektion die hauptsächliche Ursache für die Variabilität der Schnabelfärbung bei Amsel-Männchen sind.

Introduction

Parasite-mediated sexual selection theory (PMSS) predicts an inverse relationship between the amount of parasites and the expression of male ornaments (Hamilton and Zuk 1982), thereby explaining the fitness benefits females acquire by selecting more mates with evolved ornaments. Carotenoid-based ornaments in birds take the form of yellowish to reddish colouration in feathers and integuments and are known to play an important role in sexual selection (Andersson 1994; Hill and McGraw 2004). In addition to their ornamental value, carotenoids also function in a number of physiological roles, such as the protection and maintenance of the reproductive function, the scavenging of free radicals and the stimulation of the immune system (Lozano 1994; Olson and Owens 1998; Alonso-Álvarez et al. 2008). Given that they are ingested as part of the bird's diet (Goodwin 1984), a trade-off between the physiological and ornamental use of carotenoids has been proposed (Hill 1999; Hill and McGraw 2006; Alonso-Álvarez et al. 2008). Recently, however, a physiological cost of an excess of carotenoids has been found to exist (Huggins 2008), which is linked to individual oxidative stress levels (Mougeot et al. 2010; Vinkler and Albercht 2010). Carotenoids have been shown to be related to circulating levels of a number of biochemical indicators of physiological status (Huggins 2008). Consequently, unlike in feathers, the expression of carotenoids in integuments is dynamic and may respond to changes in individual condition (Faivre et al. 2003a; Pérez-Rodríguez and Viñuela 2008). Predictions of PMSS have been tested in carotenoid-coloured integuments (Figuerola et al. 2005; Martínez-Padilla et al. 2007; Baeta et al. 2008; Mougeot et al. 2010), and the colouration of ornaments has been found to be related to indicators of health (McGraw and Ardia 2003; Bertrand et al. 2006; Mougeot 2007; Mougeot et al. 2007, 2009) and reproductive ability (Massaro et al. 2003; Peters et al. 2004; McGraw et al. 2005; Hill and McGraw 2006).

The Common Blackbird (Blackbird, *Turdus merula*), a common medium-sized Palaearctic passerine, has become a model for the study of carotenoids in birds. Males have melanin-pigmented plumage and a carotenoid-pigmented bill that ranges in colour from pale dirty yellow to bright orange (Cramp and Perrins 1994). The bill colouration of the male Blackbird has been found to be related to reproductive abilities (Faivre et al. 2001; Préault et al. 2005), survival (Gregoire et al. 2004), physical development (Bright et al. 2004) and immune status (Faivre et al. 2003a, b). Baeta et al. (2008) showed that individuals with good access to carotenoids in their diets had more colourful bills and a slower replication of the intestinal parasite *Isospora*. However, the relationship between parasites and bill colouration in male Blackbirds remain controversial. A number of studies have found no relationship between Blackbird bill colouration and the presence or intensity of parasites in the wild (Hatchwell et al. 2001; Bright et al. 2004), although a recent study performed in captivity found a negative relationship between bill colouration and relative abundance of parasites (Biard et al. 2010). Most studies performed to date have focussed on a single parasite species, and only a few have examined different species of parasites. A possible explanation for these different results is the complexity of parasitic aggregation patterns, which hinders the identification of the potential relationships between colouration and parasites when only a single group of parasites is studied. Furthermore, none of the previous studies on carotenoid-based pigmentation have taken into account the biochemical parameters of the physiological variables that could interact with parasitisation in the regulation of the expression of ornamental carotenoids. Plasma biochemical characterisation provides relevant information on the nutrition and condition of the organism or of different organs and, although widely used in clinical diagnosis, is employed only rarely in studies on behavioural ecology (but see, for example, Alonso-Álvarez et al. 2002; Hegyi et al. 2010).

The aims of this study were to examine (1) how biochemical parameters, haematological values and endoparasites (including both blood and celomic parasites) are related to bill colouration and (2) how parasite richness and abundance are related to biochemical and haematological values, and to explore (3) possible patterns of parasitic aggregation in wild male Blackbirds during the breeding season.

Methods

Field work

A total of 54 male Blackbirds were trapped between March and May in 2004 and 2005 in the city of Seville (37°23′11″N, 5°57′46″W) using mist nests. All birds were individually marked with numbered aluminium rings and their body mass (to the nearest 0.5 g) and wing length (maximum chord to the nearest 0.5 mm) measured. The ages of the captured birds were determined using the method of Svensson (1984) for first year or adult birds. All individuals were manipulated in the same way in order to minimise potential differences in the corticosterone-mediated alteration in measured biochemical values (Müller et al. 2006). Birds were kept individually in cloth bags for 20 min to collect faecal samples (n = 42 individuals). Droppings were immediately placed in individually marked vials containing 5% formol; the collection time was recorded for each sample. Subsequently, 500 µl of blood was taken from the jugular vein using 29 G sterile insulin syringes, following which the birds were released back into the wild. A drop of blood was smeared on a microscopy slide (Bennett 1970), air dried and stained using Diff-Quick solution. The remainder of the blood sample was placed in a vial and within a few hours centrifuged (10 min, 6,000 rpm; Minispin centrifuge; Eppendorf, Hamburg, Germany) to separate serum. Sera were frozen at −20°C for a maximum of 1 month until subsequent analysis. The bill colour of all 54 individuals was measured using a MINOLTA 2600 spectrometer (Minolta, Osaka, Japan), which uses a high-energy xenon flash illumination and a dual-40-element silicon photodiode array. Colour was determined under both condition 1 (curves between 360 and 740 nm in 10-nm steps) and condition 2 (light, chroma and hue). Automatic calibration was performed before all colour determinations. Each determination consisted of six measurements (three in condition 1 and three in condition 2), and the mean value of each condition was used in the final analysis.

Haematology and coprology methods

Samples of droppings were filtered through a double lint cloth and then homogenised to obtain a dilution that was examined for parasite eggs and coccidian oocysts in a McMaster chamber (as per Williams 1973). To avoid dilution bias, 200 µl of the same dilution was subsequently taken from the chamber and dried in a 54°C heater; the resulting extract was weighed to the nearest 0.0001 g. The number of parasite eggs or oocysts per milligram of dry extract of faeces was obtained by dividing the number counted in the chamber by the estimated mass of the scanned sample (see López et al. 2007 for details). The most prevalent parasites were protozoan coccidian *Isospora* (prevalence: 60%) and an unidentified species of cestode (23%), which were included independently in subsequent analyses. The remaining parasites (other cestodes, strigeids and *Ascarididae*, *Spiruridae* and *Syngamidae* nematodes) were found at prevalences of <12% and were included together in the analysis as a variable coded as ‘other parasites’. The blood smears were examined for the presence of blood parasites at high magnification (oil immersion, 1,000×; 15,000 erythrocytes/sample; see Godfrey et al. 1987). *Haemoproteus* spp., *Plasmodium* spp. and *Leukocytozoon* spp. were detected, with prevalences of 50, 9 and 19%, respectively. Infection intensity could only be estimated in the intra-erythrocytic species *Haemoproteus* and *Plasmodium.* The white blood cell count (WBC) was estimated by counting the number of leukocytes on 20 similar-density light microscope fields (n = 41 blood smears, 400×) and multiplying this value by 100 (Wiskott 2002). The cellular type (as per Campbell 1995) of 100 leukocytes was estimated at 1,000× magnification. The hertophil to lymphocyte ratio (H/L), which is considered to be a reliable assessor of stress in birds (Davis et al. 2008), was calculated as the percentage of heterophils divided by the percentage of lymphocytes.

Serum biochemistry

Twelve different plasma biochemistry variables were measured from 33 serum samples collected in 2005. The samples were thawed and quantitatively assayed for aspartate aminotransferase (AST), bile acids (BA), creatinine kinase (CK), uric acid (UA), glucose (Glu), phosphorous (Phos), calcium (Ca), total proteins (TP), albumin (Alb), globulin (Glob), potassium (K) and sodium (Na) using a Vetscan (Abaxis, Union City, CA) dry and liquid biochemistry-based analyser that performs both standard and reference analyses. Each sample was analysed in a rotor containing dry test-specific reagent beads with blank reagents to calculate several determinations (see Table 1; for more information see, for example, Tedokon et al. 1992; Van Pelt 1994; Stevens 1996 and Puri 2006). The mean serum biochemistry values obtained are shown in Table 1. Given that CK has been shown to dramatically increase after bird capture (Guglielmo et al. 2001), and that we kept the birds for 20 min following capture in ringing bags, the high values of this parameter in our sample may not be representative of those observed in birds just after capture. However, the handling time was very similar for all birds.

Colour measurements

The bill carotenoid chroma of the bill colouration was determined by dividing the difference between reflectance at 700 and 450 nm by the reflectance at 700 nm (see Montgomerie 2006, 2008). The specific chroma parameter was selected because it only expresses the reflection in the orange section of the spectrum. Moreover, lightness and hue values obtained in the condition-2 determinations were included in subsequent analyses.

Statistical analysis

The WBC, Na and AST values were log-transformed [Log (value + 1)] to fit a normal distribution, while H/L, BA and Ca were ranked because there were no common

Table 1 Mean values and 95% confidence interval (CI) for the 12 biochemistry parameters evaluated in the apparently healthy male Blackbirds selected in the study

Parameter	Method[a]	Mean	95% CI
Aspartate aminotransferase (U/l)	Modified IFCC reference	691.58	535.19–847.96
Bile acids (μmol/l)	Thio-NAD+	1.12	0–2.39
Creatinine kinase (U/l)	Modified IFCC reference	5649.70	4269.92–7029.47
Uric acid (mg/dl)	UA-specific uricase	10.85	9.40–12.29
Glucose (mg/dl)	Copper-reduction	352.03	333.04–371.02
Inorganic phosphorous (mg/dl)	SP + PGM and G-6-PDH	2.09	1.59–2.58
Calcium (mg/dl)	Arsenazo III	8.87	8.45–9.29
Total protein (g/dl)	Cupric ionisation	4.16	3.93–4.38
Albumin (g/dl)	Bromcresol Green	2.62	2.50–2.75
Globulin (g/dl)	Difference TP-ALB	1.54	1.29–1.79
Potassium (mmol/l)	Pyruvate kinase	4.62	3.91–5.34
Sodium (mmol/l)	ONPG	156.36	153.49–159.23

[a] Methodology employed for every determination is specified: IFCC, International Federation of Clinical Chemistry; UA, uric acid; SP + PGM + G-6-PDH, sucrose phosphorylase + phosphoglucomutase + glucose-6-phosphate dehydrogenase; ONPG, ortho-Nitrophenyl-β-galactoside; TP, total proteins; ALB, albumin

transformations that normalised the data (Conover and Iman 1981). Body condition was estimated as the residuals of the linear regression between body mass and wing length (see Schulte-Hostedde et al. 2005).

A principal component analysis (PCA) was performed using the 12 biochemical variables to identify the principal axis of variation. Five components with eigenvalues >1 were obtained, which explained 76.8% of the variance (Table 2). PCB1 was mainly influenced by Glob and TP. Given that the TP value reflects metabolic homeostasis in birds and variations in Glob values are related to homeostasis alterations (Fudge 1996), PCB1 was considered to be an indicator of general health status. PCB2 was mainly influenced by AST and CK. Because both parameters are directly related to muscle fibre activity in homeostatic birds (Fudge 1996; Stevens 1996), PCB2 was considered as a variable related to muscular activity. PCB3 was mainly affected by Na and K, which are responsible for the homeostatic exchange of fluids between the intracellular and the extracellular compartments (alterations in the Na–K balance are related to failures in the fluid homeostasis) (Hochleithner 1994; Stevens 1996). Hence, PCB3 was considered to be an indicator of fluid balance. PCB4 was mainly influenced by Ca and Alb. As Ca is mainly related to membrane permeability in healthy birds and Alb, under homeostatic conditions, is related to oncotic pressure (Stevens 1996), PCB4 was considered to be a variable related to cell hydration level. PCB5 was influenced by UA and Phos. UA is influenced by intake in healthy birds, whereas Phos is derived from the diet and plays an important role in the storage, release and transfer of energy (Hochleithner 1994); thus, PCB5 was considered to be a negative indicator of nutritional status.

Similarly, parasitation values were analysed using a PCA to identify the principal axis of variation in our sample. If a pattern of parasite aggregation were to be present, we would expect to find a component that was significantly influenced by several groups of parasites. Three significant components were obtained, which explained 64.5% of the variance (Table 3): PCP1 was negatively influenced by *Leukocytozoon* prevalence and positively influenced by *Haemoproteus* and the 'other parasite' loads; PCP2 was positively influenced by *Isospora* and the cestode loads; PCP3 was positively influenced by the *Plasmodium* load.

Relationships between parasite and haematological variables, biochemistry variables and bill colouration were analysed using multivariate general linear models (SPSS ver. 13.0 package; SPSS, Chicago, IL). All of the two-way interactions between covariates and with factors were included in the models, and stepwise backwards selection procedures were followed until all of the independent variables remaining in the model had significantly increased ($P < 0.05$) the fit of the model. Given the different sample size of our groups of variables, different multivariate analyses of variance (MANOVAs) were used to avoid losing cases. Due to the occurrence of circadian oocyst shedding in passerine *Isospora*, a morning/afternoon factor was included in all models that included *Isospora* load (see López et al. 2007). We first investigated how biochemistry components, parasite components, body condition and haematological values were related to bill colouration. We then

Table 2 Biochemistry parameters evaluated in the selected Blackbirds, the part of the body in which they are active, their functional relevance according to Hochleithner (1994), and the rotated component matrix (Varimax rotation with Kaiser normalisation) of the PCA

Parameter	Activity	Evaluation	Principal components				
			PCB1	PCB2	PCB3	PCB4	PCB5
Aspartate Aminotransferase	Liver and muscle	Muscular activity/damage liver structural damage	0.41	*0.73*	−0.02	−0.31	−0.21
Bile acids	Intestine	Liver functioning	−0.44	0.59	−0.02	0.24	0.38
Creatinine kinase	Striated muscle	Muscular activity/damage	−0.03	*0.90*	0.05	−0.03	0.02
Uric acid	Catabolic in liver and kidney	Nutrition status renal damage	0.12	−0.12	−0.41	−0.11	*0.71*
Glucose	All of the body	Sepsis alimentary status pancreatic functioning	0.01	0.13	−0.07	0.54	−0.23
Inorganic Phosphorous	All of the body	Nutritional status renal functioning	0.26	0.15	0.29	−0.29	*0.68*
Calcium	Bone, nervous tissue and cell membranes	Nutritional status fluid balance renal functioning	0.26	−0.30	−0.03	*0.78*	0.05
Total protein	All of the body	Metabolic homeostasis kidney functioning	*0.91*	0.02	−0.05	0.31	0.13
Albumin	All of the body	Liver functioning kidney functioning cell hydration	−0.14	−0.08	0.48	*0.76*	−0.13
Globulin	All of the body	Metabolic homeostasis fluid balance	*0.90*	0.03	−0.28	−0.10	0.19
Potassium	All of the body	Cell lysis osmotic balance	−0.07	0.25	*0.87*	−0.04	0.11
Sodium	All of the body	Osmotic balance antigenic stimulation kidney functioning	−0.18	−0.23	*0.74*	0.09	−0.15

PC, Principal component analysis; B1–B5, Biochemistry components

Variables which accounted for >0.6 are given in italics

Table 3 Rotated component matrix (Varimax rotation with Kaiser normalisation) of the PCA performed on the parasite burdens/prevalence and haematological values of the sampled Blackbirds

Component	PCP1	PCP2	PCP3
Log (*Plasmodium* burden + 1)	0.05	−0.09	*0.89*
Haemoproteus burden	*0.63*	0.11	−0.23
Leukocytozoon prevalence	*−0.64*	0.26	−0.06
Log (*Isospora* burden + 1)	−0.42	*0.64*	0.41
Log (Cestode burden + 1)	0.16	*0.84*	−0.21
Log (Other parasite burden + 1)	*0.67*	0.16	0.36

P1–P3, Parasite components

Variables which accounted for >0.6 are given in italics

conducted the following ANOVAs: (1) bill chroma, lightness and hue were included as the dependent variables, with biochemistry components and body condition as covariates and age as a factor; (2) bill chroma, lightness and hue were included as the dependent variables, with parasite components and body condition as covariates and year, morning/afternoon and age as factors; (3) bill chroma, lightness and hue were included as the dependent variables, with H/L, WBC and body condition as covariates and age as a factor. Secondly, we analysed the relationships between parasite components and biochemical components, body condition and haematological values. The following MANOVAs were performed: (1) parasite components were included as dependent variables, with biochemistry components and body condition as covariates and age and morning/afternoon as a factor; (2) parasite components were included as dependent variables, with H/L, WBC and body condition as covariates and age, year and morning/afternoon as factors.

Results

Patterns of parasitic aggregation

Our results do not show any general pattern of aggregation in the different types of parasites in the individuals sampled. which means that the different parasite species analysed in our study were parasitising different individuals. Interestingly, haemoprotozoa *Haemoproteus* and the group ‘other parasites’ were related and positively accounted for PCP1, which might imply (1) the possibility that *Haemoproteus* affects host condition in a way that favours parasitisation by other parasite species or (2) a possible association between *Haemoproteus* and one of the parasite species included in the second group. Unfortunately, our sample size did not allow us to investigate this relationship in greater depth. *Leukocytozoon* prevalence was negatively related to both groups of parasites, indicating that individuals infected by *Haemoproteus* and 'other parasites' were not infected by *Leukocytozoon*. PCP2 showed an aggregation trend between the intestinal coccidia *Isospora* and the common cestode found in our sample, possibly because infection by one of the species may favour infection by the other.

Relationships between biochemical components and bill colouration

Body condition and the interactions between body condition and PCB1, PCB4 and PCB5, respectively, as well as the interactions between PCB1 and PCB4, and PCB3 and PCB4, and PCB3 and PCB5, respectively, were significantly related to bill carotenoid chroma (Table 4). Interestingly, none of the biochemical components independently showed a significant relationship with bill chroma. Lightness and hue did not show any significant relationship with any of the variables.

Relationships between parasite components and bill colouration

Contrary to expectations, none of the parasite components were related to any determination of bill colouration.

Relationships between haematological values and bill colouration

The haematological value H/L was significantly related to bill chroma ($F_{1,39} = 4.44$; $P = 0.04$). Individuals with high values of H/L had paler bills than those with low values of H/L. No other significant relationships were found in this model.

Relationships between biochemical and parasite components

The interaction between PCB2 and PCB5 was significantly related to PCP2 ($F_{1,22} = 5.12$; $P = 0.03$) and PCP3 ($F_{1,22} = 4.87$; $P = 0.04$) given that high values of PCB2 and PCB5 were related to low values of PCP2 and PCP3; that is, parasitation by *Isospora*, the quantified cestode and *Haemoproteus* was related to lower muscular activity and poorer nutritional status.

Relationships between haematological values and parasite components

Haematological parameters did not show any significant relationship with parasite components.

Table 4 Results of the stepwise backwards selection procedure ANOVA analysing relationships between bill colour determinations and biochemical components

Source[a]	Dependent variable	*df*, error	*F*	*P*
PCB1	Chroma	1, 21	0.01	0.94
	Lightness	1, 21	0.07	0.80
	Hue	1, 21	0.00	1.00
PCB3	Chroma	1, 21	0.02	0.88
	Lightness	1, 21	0.68	0.42
	Hue	1, 21	7.93	0.01
PCB4	Chroma	1, 21	0.37	0.55
	Lightness	1, 21	0.02	0.89
	Hue	1, 21	3.56	0.07
PCB5	Chroma	1, 21	0.88	0.36
	Lightness	1, 21	0.13	0.72
	Hue	1, 21	1.37	0.26
Body condition	Chroma	1, 21	6.37	0.02
	Lightness	1, 21	0.13	0.72
	Hue	1, 21	0.14	0.71
PCB1 × PCB4	Chroma	1, 21	5.50	0.03
	Lightness	1, 21	0.01	0.93
	Hue	1, 21	1.91	0.18
PCB3 × PCB4	Chroma	1, 21	13.36	<0.01
	Lightness	1, 21	0.42	0.53
	Hue	1, 21	0.49	0.49
PCB3 × PCB5	Chroma	1, 21	23.86	<0.01
	Lightness	1, 21	0.08	0.79
	Hue	1, 21	0.41	0.53
PCB1 × body condition	Chroma	1, 21	9.75	0.01
	Lightness	1, 21	0.09	0.76
	Hue	1, 21	0.05	0.83
PCB4 × body condition	Chroma	1, 21	11.56	<0.01
	Lightness	1, 21	0.02	0.88
	Hue	1, 21	2.65	0.12
PCB5 × body condition	Chroma	1, 21	10.64	<0.01
	Lightness	1, 21	0.00	0.95
	Hue	1, 21	3.69	0.07
PCB2	Chroma	1, 20	0.54	0.47
	Lightness	1, 20	0.05	0.83
	Hue	1, 20	0.00	0.99

ANOVA, Analysis of variance

Discussion

Our results are the first to demonstrate a relationship between biochemical indicators of health status and orange bill coloration in male Blackbirds. Interestingly, none of the principal components alone was significantly related to bill colouration, although some of the interactions between these were. First, the interaction between the component indicating general health status and that indicating cell hydration level was negatively related to bill chroma. In other words, the orange in male Blackbird bills decreased with increasing levels of the biochemical indicators of general health failure and cell dehydration. Thus, alterations in general homeostasis due to dehydration provoked a paler bill colouration. This result indicates that bill colouration was not affected by either alterations in homeostasis due to causes other than dehydration nor by dehydration stages that did not affect general homeostasis. Second, the interaction between the component indicating cell hydration level and that indicating fluid balance was negatively related to bill chroma. That is to say, the orange in bills decreased with increasing general dehydration. Given that the principal components analysed here may be affected by different parameters, this result suggests that only alterations in both components concurrently (i.e.those alterations exclusively related to dehydration) have the potential for affecting bill colouration. Third, the interaction between the component indicating cell hydration levels and that indicating nutritional status was negatively related to bill chroma. This means that when biochemical indicators of both malnutrition and cell dehydration increased, the orange in male Blackbird bills decreased. This result links the alterations of two principal components that are provoked by the same cause, namely, a reduced intake, which causes alterations related to both malnutrition and dehydration. The component accounting for muscular activity did not show any relationship with bill colouration. The potential differences in CK generated by time of bleeding after capture (Guglielmo et al. 2001) fall within this category. Interestingly, we found a significant relationship between bill colouration and the body condition index measured in the field, with males having a good body condition displaying more colourful bills than males with a poor body condition. Bright et al. (2004) reported similar results, as did Jawor et al. (2004) and Pérez-Rodríguez and Viñuela (2008) in other studies of carotenoid-coloured integuments conducted in different species. The interaction between body condition and the components indicating general health status, cell hydration level and nutritional status were also related to bill chroma. Consequently, changes in body condition due to both dehydration and malnutrition were reflected in paler bill colourations. Similarly, only alterations in general homeostasis that affected the body condition provoked paler bills. This finding agrees with the handicap theory (Zahavi and Zahavi 1997), which predicts that only the best-condition individuals can afford the energetic cost of spending more carotenoids on ornamental functions. Taken together, these results demonstrate that the colouration of the male Blackbird bill has the potential to convey information on current physiological status. They also that nutrition and hydration are the main parameters affecting bill colouration.

The predictions of the PMSS were not supported by the results of our study since no significant relationship was found between bill chroma and any of the components accounting for the parasites evaluated. As such, our result agrees with those of previous studies that failed to identify any relationship between parasites and bill colouration in male Blackbirds (Hatchwell et al. 2001; Bright et al. 2004). In contrast to these results, a negative relationship between certain groups of parasites and the colouration of carotenoid-coloured integuments has been pointed out in captive Blackbirds (Biard et al. 2010) and in other species (Mougeot et al. 2005; Martínez-Padilla et al. 2007). These different outcomes may possibly be due to (1) differences in the parasite species and community infecting the hosts in different locations, (2) inter-individual host differences in physiological response or (3) the different effects that diverse type of parasites have on host physiology. In this context, we found that only *Isospora*, the unidentified cestode and *Leukocytozoon* were related to biochemistry values, with all three being related to low muscular activity and poor nutritional status. This finding suggests that, of the parasite species evaluated, these three species may be the only type of parasites affecting host physiology. Moreover, none of the parasites showed any relationship with the other biochemical values or with haematological values. In line with this finding, the parasites in our sample did not show any clear aggregation patterns other than those between *Isospora* and the unidentified cestode. This is an important result given the traditional focus on a single or small number of parasite species. Several studies have demonstrated that the intensity of infection by different species of parasites is not strongly correlated at the intraspecific level (Møller 1991; Weatherhead et al. 1993). Our results show that, in many cases, the parasitic loads of different groups of parasites are not related; consequently, it is not possible to derive an 'index of parasitism' based on a small number of pathogens and, therefore, conclusions obtained for one group of parasites cannot be extrapolated for the full community of parasites. This limits our capacity to rigorously test parasite-mediated selection hypotheses unless clear indications of the effects on host fitness exist for a significant fraction of the parasite community. We found a negative relationship between the H/L ratio and the orange colour of male Blackbird bills. Given that the H/L ratio has been described as an indicator of stress (Davis et al. 2008), our results suggest that birds with greater stress levels have paler bills than those with lower stress levels which, in turn, provides evidence for a relationship between the orange in bills and health constraints (Faivre et al. 2003a; Baeta et al. 2008). Despite previous studies that have demonstrated a link between Blackbird bill colouration and the immune system (Faivre et al. 2003a; Baeta et al. 2008), our study failed to find a relationship between bill colouration and WBC. Underlying this result, high WBC values may indicate either (1) potent immune abilities or (2) immune stimulation by infection (Norris and Evans 2000), and observational studies cannot differentiate between these possibilities. Finally, despite lightness and hue have shown relevant physiological implications in other studies (see Hill and McGraw 2006), these colour measurements were not significantly related with any of the variables studied here.

In conclusion, our results support the premise that carotenoids are involved in a trade-off between their use for ornamentation and their requirement to satisfy physiological needs, and that the colouration of male Blackbird bills signals its immune and nutritional status. As the colouration of integuments in birds is a dynamic ornament, carotenoid content is likely to be a valid indicator of the actual physiological status of an individual bird. In line with results from previous studies, our results failed to find a relationship between bill colouration and parasite burden or prevalence, probably due to the complex relationships between parasites, health status and carotenoid-based ornamentation. We therefore conclude that biochemistry parameters related to different aspects of health status may help provide a better understanding of the regulation of ornament expression in birds.

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Declaration All of the work performed in the study reported here complies with the current Spanish laws.

Conflict of interest The authors declare that they have no conflict of interest.

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