Oecologia (2010) 162:825–835
DOI 10.1007/s00442-009-1510-y

PHYSIOLOGICAL ECOLOGY - ORIGINAL PAPER

Carotenoid-based plumage colouration is associated with blood parasite richness and stress protein levels in blue tits (*Cyanistes caeruleus*)

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Received: 30 December 2008 / Accepted: 30 October 2009 / Published online: 25 November 2009
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Abstract Carotenoids are molecules that birds are not able to synthesize and therefore, must be acquired through their diet. These pigments, besides their function of giving birds red and yellow colouration when deposited in feathers, seem to act as immune-stimulators and antioxidants in the organism. Hence, only the healthiest individuals would be able to express carotenoid-based ornaments to a larger extent without compromising the physiological functions of carotenoids. Various studies have reported that birds infected by parasites are paler than those uninfected, but, to our knowledge, none of them has assessed the possible effect of multiple infections by blood parasites on plumage colour. By comparing the yellow colour in the breast plumage of blue tits, *Cyanistes caeruleus*, between birds infected by different numbers of blood parasite genera, we found that those birds infected by more than one genus were paler than those parasitized just by one. In addition, we examined the potential role of carotenoid-based plumage colour of blue tits as a long-term indicator of other parameters of health status, such as body condition and immunoglobulin and heat shock protein (HSP) levels. Our results indicate that more brightly coloured birds had lower HSP70 levels than paler birds, but we did not find any significant association between colour and body condition or immunoglobulin levels. In addition, we found a positive significant association between *Haemoproteus* density of infection and HSP60 levels. Overall, these results support the role of carotenoid-based colours as indicators of health status in blue tits and show detrimental effects of parasitism on this character.

Keywords Bird · Health status · Heat shock proteins · Multiple infections · Yellow breast

Communicated by Heli Siitari.

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Introduction

Numerous observational and experimental studies have shown that female birds prefer males with the brightest and most intense plumage colours (e.g. Zuk et al. 1990; Hill 1991; MacDougall and Montgomerie 2003), although the proximate reasons for this choice are not fully understood. One of the potential selective pressures proposed to explain the evolution of female mate choice is that plumage colour may signal health status and parasite resistance (Hamilton and Zuk 1982); thus, females would acquire selective advantages by mating with colourful males. Several hypotheses have been developed in this respect. The influential

hypothesis of Hamilton and Zuk (1982) posits that females choosing a brightly coloured male may gain benefits for their nestlings in the form of heritable resistance to parasites. Also, females may avoid parasite transmission to themselves and their nestlings (Clayton 1990) or they may benefit from a higher parental effort performed by a healthy male (Read 1990). Despite the role of colourful plumage having been studied especially in males, it is known that females also show conspicuous plumages in several species. Traditionally, it has been assumed that this might be due to a genetic correlation with male ornamentation, thereby suggesting that female colouration was not functional (Lande 1980). However, males may suffer costs associated with reproduction and a way to reduce these costs would be choosing a showy female if showiness signals her good quality (Amundsen 2000). Indeed, evidence is accumulating on the adaptive function of female ornamentation (see Kraaijeveld et al. 2007 for a review).

Carotenoid-based colours are very common among birds (Goodwin 1984). It has been proposed that the honesty of carotenoid-based ornaments as quality signals is based on the multiple functions of carotenoids in the organism. Besides being deposited in feathers and thus giving yellow and red colours to birds' plumages, they seem to participate as immune-stimulators and antioxidants in the organism (Lozano 1994; Møller et al. 2000). Among their functions we can cite their role as scavengers of free radicals and immunosuppressive peroxides (e.g. Chew 1993; Surai and Speake 1998; von Schantz et al. 1999; Møller et al. 2000; but see Costantini and Møller 2008), as well as enhancers of the production of lymphocytes, the phagocytic ability of neutrophils and macrophages, and of tumour immunity (Møller et al. 2000; Surai et al. 2001). In addition, carotenoids are pigments that vertebrates cannot synthesize de novo, so they must be obtained through the diet (Hill 1992; Olson and Owens 1998). Hence, according to Hamilton and Zuk (1982), only individuals of high quality (i.e. those with higher resistance to parasites and/or higher foraging capacity) would be capable of being intensely coloured without compromising the quantity of carotenoids allocated to other physiological functions.

The negative effects of blood parasites on plumage colours have been reported in previous studies (Weatherhead 1990; Sundberg 1995; Merilä et al. 1999; Hõrak et al. 2001), those birds heavily infected by parasites being normally less pigmented than lightly infected ones, and on the whole, parasitized birds being paler than unparasitized ones. However, these studies on the effects of blood parasites on plumage colour are based on only one parasite species, whereas, to our knowledge, there is no study examining the possible effect of joint infections by multiple blood parasites on carotenoid-based plumage colour. Such studies may contribute to a better understanding of the role of carotenoid-based plumage colourations as quality signals, since birds in the wild are normally infected by different parasite species simultaneously (Merino et al. 2000; Valkiūnas et al. 2003). Furthermore, it has been shown that hosts can suffer more severe harmful effects, like anaemia (Graham et al. 2005), loss of body mass (Evans and Otter 1998; Graham et al. 2005; Marzal et al. 2008), production of a less saturated carotenoid-based colouration (Brawner et al. 2000) and reduction of survival (Evans and Otter 1998; Davidar and Morton 2006; Arriero and Møller 2008; Marzal et al. 2008) due to multiple simultaneous infections, if compared to single ones.

The aim of this study was to evaluate the role of a carotenoid-based trait, the yellow breast plumage colouration of blue tits (*Cyanistes caeruleus*), as a long-term indicator of health status in a population commonly infected by several species of blood parasites (Merino et al. 2000). The blue tit is a small (11 g) insectivorous passerine, very common in Europe. It is a slightly sexually dichromatic species, males being more intensely coloured than females (Cramp 1998). In blue tit males, yellow carotenoid-based colouration has been shown to indicate parental investment (Senar et al. 2002), while in females it reflects their reproductive capacity (Doutrelant et al. 2008). Moreover, there is assortative mating by this trait (Hidalgo-García 2006; Ferns and Hinsley 2008), which suggests that yellow plumage colouration might be sexually selected in both sexes. The health status variables studied here were parasite richness (number of parasite genera birds are infected with), *Haemoproteus* infection intensity, body condition index and the levels of total immunoglobulins and stress proteins (heat shock proteins; HSPs). Immunoglobulins play an important role in the specific humoral immune response of vertebrates, being responsible for antigen recognition (Roitt et al. 1996). Measures of humoral immune response are becoming widely used in many ecological studies (Norris and Evans 2000; Martínez-de la Puente et al. 2007a; Tomás et al. 2007), and fitness costs of immune defence against parasites and trade-offs in the face of limited resources have been reported (Deerenberg et al. 1997; Moreno et al. 1999; Soler et al. 2002; Merino et al. 2006; Moreno et al. 2008). HSPs are molecules that maintain cellular homeostasis by responding to a wide array of stressors, like heat (Gehring and Wehner 1995), toxins (Mariño et al. 1999), oxidant compounds (Martínez et al. 1999a), cold (Martínez et al. 2001) and parasites (Merino et al. 1998; Martínez et al. 1999b; Tomás et al. 2005). Likewise, studies on the ecological and evolutionary role of the HSP-mediated stress response have been published in recent years (see Sørensen et al. 2003 for a review).

We hypothesize that if the yellow colour is a signal of phenotypic quality, more intensely coloured birds should be lightly infected by *Haemoproteus* and they should harbour

fewer blood parasite genera. Also, colourful birds would be in better body condition and would show higher levels of immunoglobulins (immunocompetence) and lower stress protein levels.

Materials and methods

The study was carried out during the 2004 and 2007 breeding seasons in a Pyrenean oak (*Quercus pyrenaica*) forest located in Valsaín, central Spain (Segovia province, 40º53′N, 4º01′W, 1,200 m a.s.l.), where a population of blue tits breeds in wooden nest boxes. This population has been studied since 1991 (e.g. Merino et al. 2000; Martínez-de la Puente et al. 2006). Every year, nest boxes are periodically inspected to determine the laying date (day 1 = 1 April), clutch size, hatching date and fledging success.

After removing cases of birds recaptured in 2007 (five birds) to avoid pseudoreplication, we kept samples from 166 birds (85 females: 30 yearlings and 55 adults, and 81 males: 34 yearlings and 47 adults) in 2004 and 58 birds (29 females: 9 yearlings and 20 adults and 29 males: 14 yearlings and 15 adults) in 2007.

In both study years, adult birds were trapped in nest boxes twice. In the first capture (when nestlings were 3 days old) they were ringed if necessary, weighed with an electronic balance to the nearest 0.1 g and sampled for blood (see below). In addition, 0.1 mg of an antimalarial drug, primaquine, diluted in 0.1 ml of saline solution or the same volume of saline solution was injected subcutaneously into the abdominal region for another experimental purpose (see Martínez-de la Puente et al. 2006, 2007b; Tomás et al. 2008). During the second capture (10 days later), tarsus length was measured with a digital calliper to the nearest 0.01 mm. Birds were sexed and classified as yearling or older (≥2 years) according to plumage characteristics as described by Svensson (1992). Breast yellow plumage colour was objectively measured using a portable spectrophotometer (CM-2600d, Minolta), that covers the range of wavelengths between 360 and 740 nm. In order to prevent the incidence of ambient light the spectrophotometer measuring mask was placed perpendicularly against one flank of the breast feather surface to take colour measures while holding the bird horizontally. From the raw spectral reflectance data, we computed the "carotenoid chroma" $(R_{700}-R_{450})/R_{700}$ for each individual (Andersson and Prager 2005). This is the relative difference in reflectance between the wavelengths of minimum (700 nm) and maximum (450 nm) absorbance of the two main carotenoids in blue tit plumage (lutein and zeaxanthin). This measure has the advantage of being a strong correlate of perceived chroma and also the best spectrometric estimate of actual carotenoid concentration (Andersson and Prager 2005). During 2004, two consecutive colour measurements were taken from some individuals and the values of carotenoid chroma from these measurements were averaged to be used in the final analyses. As our methods of colour estimates had reasonably low measurement error to sufficiently describe inter-individual differences in carotenoid chroma [repeatability (Lessells and Boag 1987) of two consecutive measurements $R_i = 0.66$, $F_{13,14} = 4.83$; $P < 0.01$], just one spectral measurement was taken from each individual in 2007. As in other studies based on the use of spectrophotometers not including UV reflectance (Figuerola et al. 1999; Senar et al. 2002; Hidalgo-García 2006; Ferns and Hinsley 2008), we face a potential problem since blue tit visual perception extends into the UV range of the spectrum (Hunt et al. 1998; Örnborg 2002). However, our main aim is to explore whether carotenoid chroma as experienced by human observers is associated with some health parameters of birds. Therefore, objective measurements of chroma in the human-visible part of the spectrum appear adequate for our purposes.

For the molecular detection of blood parasites (*Haemoproteus*, *Leucocytozoon*, *Plasmodium*, *Lankesterella* and *Trypanosoma*), a drop of blood from the brachial vein of birds captured in 2004 was stored at −20°C until processed, whereas samples of 2007 were stored in FTA cards (Whatman, UK). DNA was extracted using a commercial kit (UltraClean DNA BloodSpin kit; MO BIO Laboratories, Calif.) or, in the case of FTA cards, by applying the protocol described in Merino et al. (2008). In both cases we immediately amplified the cytochrome B gene using the primers described in Table 1. Polymerase chain reactions (PCRs) used 25-µl reaction volumes containing 20 ng template DNA, 50 mM KCl, 10 mM TRIS–HCl, 1.5 $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, Calif.). The reactions were cycled under the following conditions using a thermal cycler (MasterCycler Personal, Eppendorf): 94°C for 10 min (polymerase activation), 40 cycles at 95°C for 40 s, annealing temperature for 1 min (see Table 1), 72°C for 1 min, and a final extension at 72°C for 10 min. In some cases there was not enough blood to detect the presence of all parasite genera in samples from 2004; therefore, we used data obtained from microscopical observations of blood smears in these cases. Blood smears were prepared immediately upon extraction and were air-dried, fixed in absolute ethanol and stained with Giemsa stain for 45 min. Half of a smear was scanned under an optical microscope at 200× to search for large parasites such as *Trypanosoma* and *Leucocytozoon*, whereas small intra-erythrocytic parasites, such as *Haemoproteus, Lankesterella* and *Plasmodium* were detected in the other half of the smear at 1,000× with the oil immersion objective (see Merino et al. 1997). In spite of the different efficiency of

Table 1 Sequences and annealing temperatures of the primers

Primer	Annealing	Parasite	Sequence (5′→3′)
LDLd	58	*Leucocytozoon*	CAT TCY ACW GGT GCA TCT TT
LDRd	58	*Leucocytozoon*	CTG GAT GWG ATA ATG GWG CA
PLAS-F	60	*Plasmodium*	GTA ACA GCT TTT ATG GGT TAC
4292Rw[a]	60	*Plasmodium*	TGG AAC AAT ATG TAR AGG AGT
HML	58	*Haemoproteus*	GCT ACT GGT GCT ACA TTT GT
HMR	58	*Haemoproteus*	CCT AAA GGA TTA GAG CTA CC
S-755[b]	60	*Trypanosoma*	CTA CGA ACC CTT TAA CAG CA
S-823[b]	60	*Trypanosoma*	CGA AYA ACT GCY CTA TCA GC
Hep900F	58	*Lankesterella*	GTC AGA GGT GAA ATT CTT AGA TTT G
Hep1615R	58	*Lankesterella*	AAA GGG CAG GGA CGT AAT C

[a] Primer previously published by Beadell et al. (2004)

[b] Primers previously published by Maslov et al. (1996)

molecular and microscopic techniques in detection of blood parasites (higher for molecular methods in all cases: 47.5 vs. 32.3% for *Trypanosoma*; 89.3 vs. 63.5% for *Leucocytozoon*; 85.6 vs. 72.5% for *Haemoproteus*; 47.7 vs. 32.9% for *Lankesterella* and 69.9 vs. 2% for *Plasmodium* according to molecular and microscopic detection, respectively), we included data from microscopy when necessary to increase sample size. However, as this increased the possibility of including false negatives, we also conducted the analyses including only data from molecular detection. We assigned birds to the following groups depending on their blood parasite richness, i.e. the number of blood parasite genera they were infected with: (1) one blood parasite genus, (2) two blood parasite genera, (3) three blood parasite genera, and (4) four or five blood parasite genera (see Results).

The intensity of infection by *Haemoproteus* was quantified as the number of parasites per 2,000 erythrocytes in blood smears (Merino et al. 1997). However, in some cases the parasite was detected by molecular methods and not by microscopy. In order to include these cases in the analysis on intensity of infection the variable was transformed into categories according to its distribution: (1) 0–10 parasites/2,000 erythrocytes; (2) 11–20 parasites/2,000 erythrocytes; (3) 21–40 parasites/2,000 erythrocytes; (4) 41–60 parasites/2,000 erythrocytes; and (5) 61–140 parasites/2,000 erythrocytes. Infections by other parasites showed low intensities and were not quantified.

Only in 2004, part of the blood extracted was centrifuged (2,000 *g*, 5 min) with a portable centrifuge (1,201–220 V; Labnet, Woodbridge, N.J.) which separated the cellular and plasma fractions. The cellular fraction was used to determine the levels of two different HSPs, HSP60 and HSP70, through Western blot following the protocol described in Tomás et al. (2004). The plasmatic fraction was used to determine immunoglobulin levels through a direct enzyme-linked immunosorbent assay (ELISA), using a polyclonal rabbit antichicken IgG conjugated with peroxidase (Sigma A-9046; Sigma, St Louis, Mo.). For details on the methodology see Martínez et al. (2003). Body condition index was calculated as mass/tarsus length during both years.

Treatment with primaquine reduces the intensity of infection by *Haemoproteus* and the prevalence of infection by *Leucocytozoon* in blue tits (Merino et al. 2000). To avoid any confounding effect of this treatment (e.g. on blood parasite infection), data from the first capture were used in the analyses, which was available for all variables except for plumage colour and tarsus length (obtained in the second capture). It should also be noted that in 2003 and 2006, i.e. the two breeding seasons previous to those included in the present study (when birds moulted the new plumage colour was measured the following breeding season), no bird was treated with primaquine. This precludes that any relationship found between blood parasitaemias and plumage colour of birds can be associated with previous primaquine treatments.

To study the relationship of the different variables (categorical variable: blood parasite richness; continuous variables: body condition index, levels of immunoglobulin, HSP60 and HSP70) with plumage colour (carotenoid chroma), general regression models (Statistica 2001; StatSoft) were used. Year, date of measurement, sex, age and treatment were controlled for. For simplicity only two-way interactions between categorical variables were included. Models were obtained by a backward elimination procedure. In addition, we studied the variation of the intensity of infection by *Haemoproteus* when infecting a host alone or together with other blood parasite genera and its association with carotenoid chroma and other health parameters using a general linear model, controlling for year, sex and age of birds. All the variables showed a normal distribution except date of measurement which was logarithmically transformed to satisfy assumptions of normality. Differences in sample sizes reflect missing values due to, for instance, inability to obtain enough blood from all birds.

Results

The prevalence of blood parasites was considerable, *Haemoproteus* being the most frequent blood parasite (84% in 2004 and 81% in 2007), followed by *Leucocytozoon* (87% in 2004 and 57% in 2007). Other blood parasites were also present but with lower prevalences: *Trypanosoma* (29% in 2004 and 19% in 2007), *Lankesterella* (45% in 2004 and 38% in 2007) and *Plasmodium* (65% in 2004 and 36% in 2007). The number of birds in each category of blood parasite richness was: (1) 21 birds (10 in 2004 and 11 in 2007); (2) 57 birds (33 in 2004 and 24 in 2007); (3) 74 birds (60 in 2004 and 14 in 2007); (4) 69 birds (49 in 2004 and 7 in 2007 with four different parasite genera and 12 in 2004 and 1 in 2007 infected by five parasite genera). Only one bird of 2007 was unparasitized and was not included in the analyses.

The model obtained when relating carotenoid chroma to blood parasite richness and body condition is presented in Table 2. Multiple blood parasite infections were more likely to be present in paler birds (those with lower carotenoid chroma) as compared to those parasitized by just one parasite genus. Post hoc comparisons showed that carotenoid chroma was drastically lower in birds infected by more than one blood parasite [Fig. 1; Fisher least significant difference (LSD): $P < 0.05$], but did not differ between birds infected with two, three or four genera. Additionally, carotenoid chroma of birds was significantly different between years, birds of 2007 being more colourful than birds of 2004. Moreover, we found an effect of year × blood parasite richness on carotenoid chroma. Post hoc comparisons of birds infected by the same number of blood parasite genera in different years indicated that carotenoid chroma of birds infected by two or three blood parasite genera in 2004 was lower than carotenoid chroma of birds infected by the same number of blood parasite genera in 2007 (Fig. 2; Fisher LSD: $P < 0.01$). In addition, in 2004, birds infected by one blood parasite genus had significantly higher carotenoid chroma values than birds infected by two or three blood parasite genera (Fig. 2; Fisher LSD: $P < 0.05$), whereas in 2007, birds infected by one blood parasite genus were slightly more intensely coloured than birds infected by four or five blood parasite genera (Fig. 2; Fisher LSD: $P = 0.05$) but not than birds infected by two or three blood parasite genera (Fig. 2; Fisher LSD: $P > 0.05$). On the other hand, we did not find a significant effect of date of measurement, sex or age on carotenoid chroma ($P > 0.05$). However, we found an effect of the treatment and sex × age and sex × treatment interactions on plumage colour. Young females were significantly paler than adult females and young males but not than adult males, whereas untreated males were significantly more colourful than females and treated males and overall, untreated birds had higher levels of carotenoid chroma. Finally, carotenoid chroma was not significantly related to body condition or to any of the other two-way interactions ($P > 0.05$). The final

Table 2 Model obtained when exploring the relationship of carotenoid chroma of blue tit breast plumage in 2004 and 2007 with parasite richness and body condition

Effect	*df*	*F*	*P*
Year	1,205	7.132	0.008
Treatment	1,205	5.494	0.020
Blood parasite richness	3,205	4.984	0.002
Sex × age	1,205	5.374	0.021
Sex × treatment	1,205	7.295	0.007
Year × blood parasite richness	3,205	3.142	0.026

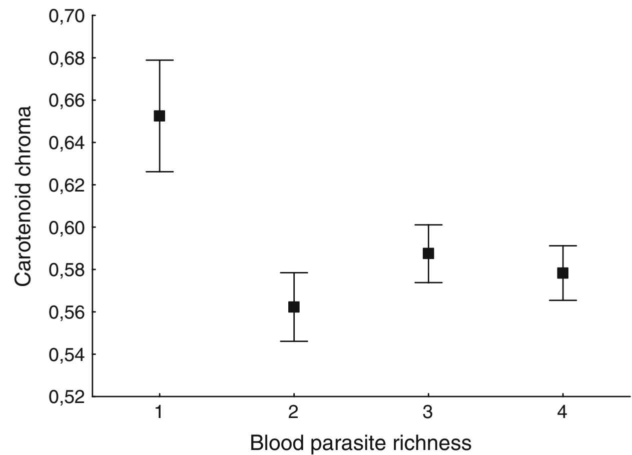

Fig. 1 Carotenoid chroma variation in relation to blood parasite richness. *Bars* indicate SE. *1* One blood parasite genus, *2* two blood parasite genera, *3* three blood parasite genera, *4* four or five blood parasite genera

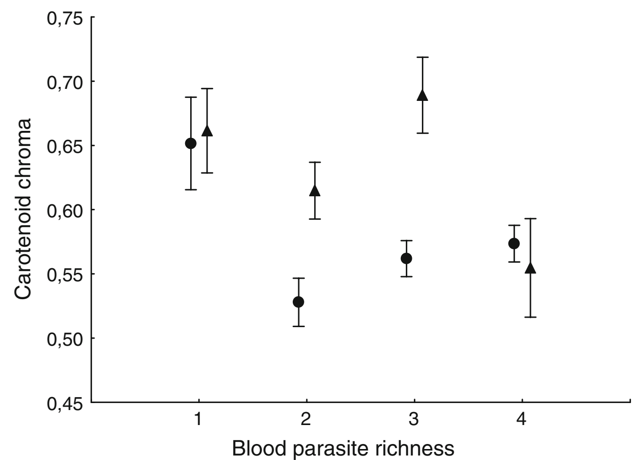

Fig. 2 Carotenoid chroma variation in relation to year × blood parasite richness interaction. *Circles* Data from 2004, *triangles* data from 2007. *Bars* indicate SE

model explained 18% of the variation in carotenoid chroma ($F_{10,205} = 5.56$, $P < 0.001$). We attained similar conclusions when analysing data based only on PCR (year, $F_{1,156} = 9.16$, $P < 0.01$; blood parasite richness, $F_{3,156} = 2.82$, $P < 0.05$; age × treatment, $F_{1,156} = 5.26$, $P < 0.05$; sex × treatment, $F_{1,156} = 5.78$, $P < 0.05$; rest of variables and interactions, $P > 0.05$). However, in this case post hoc comparisons indicated that carotenoid chroma was higher in birds infected by one blood parasite genus than in those infected by two or four genera ($P < 0.05$) but it did not differ between birds infected by one genus and three blood parasite genera ($P > 0.05$). Post hoc comparisons of the interaction age × treatment indicated that untreated adult birds were more colourful than medicated adult birds ($P < 0.01$).

Carotenoid chroma was not significantly related to immunoglobulin levels ($P > 0.05$), although higher levels of HSP70 in blood were significantly associated with a paler colour (lower carotenoid chroma) in birds (Table 3; Fig. 3). In contrast, we did not find a significant relationship between HSP60 levels and carotenoid chroma ($P > 0.05$). This model retained the effect of treatment, blood parasite richness, sex × age and sex × treatment and explained 12% of variation in carotenoid chroma ($F_{7,139} = 3.98$, $P < 0.001$). The model based only on PCR data retained the significant relationship between carotenoid chroma and HSP70 ($F_{1,99} = 7.01$; $P < 0.01$) and the effect of the interaction sex × treatment ($F_{1,99} = 4.08$, $P < 0.05$), whereas the association of chroma with the rest of the variables was not significant (all $P > 0.05$).

Finally, the intensity of infection by *Haemoproteus* in our blue tit population did not differ significantly between different categories of parasite richness ($F_{3,175} = 0.71$, $P > 0.05$) and it was not significantly associated with carotenoid chroma ($F_{1,175} = 0.03$, $P > 0.05$), body condition ($F_{1,175} = 0.20$, $P > 0.05$), immunoglobulin levels ($F_{1,119} = 0.24$, $P > 0.05$) or HSP70 levels ($F_{1,119} = 0.05$, $P > 0.05$). However, we found a positive significant association with HSP60 levels ($F_{1,119} = 10.00$, $P < 0.01$; Fig. 4). In addition, *Haemoproteus* infection intensity was higher in 2007 ($F_{1,175} = 7.08$, $P < 0.01$) and in young birds ($F_{1,175} = 25.61$, $P < 0.001$).

Table 3 Model obtained when exploring the association of levels of immunoglobulins and stress proteins with carotenoid chroma of blue tit breast plumage in 2004. *HSP* Heat shock protein

Effect	*df*	*F*	*P*
Treatment	1,139	3.935	0.049
Blood parasite richness	3,139	3.243	0.024
HSP70 levels	1,139	5.354	0.022
Sex × age	1,139	3.273	0.041
Sex × treatment	1,139	4.608	0.034

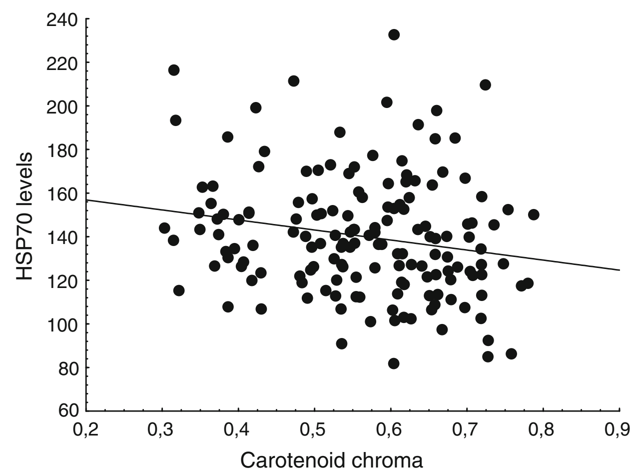

Fig. 3 Relationship between heat shock protein (*HSP*) 70 levels and carotenoid chroma of breast plumage colour of blue tits in 2004

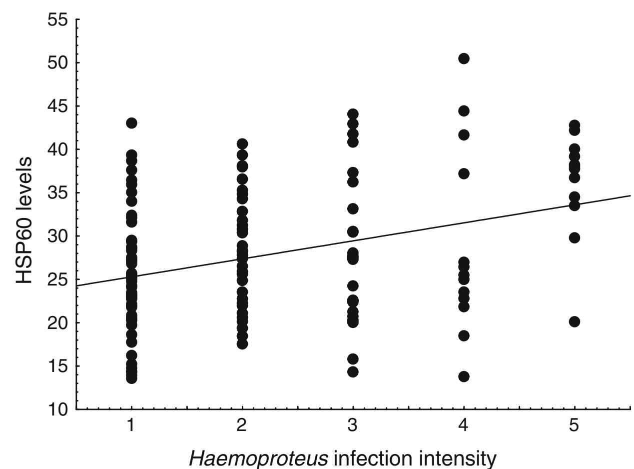

Fig. 4 Relationship between HSP60 levels and *Haemoproteus* infection intensity of blue tits in 2004. Intensity of infection was transformed to include data of infections only detected by molecular methods (see text)

Discussion

The most novel result of this study is the relationship of multiple blood parasite infections with carotenoid chroma. Birds parasitized by a single genus of blood parasite had higher carotenoid chroma and thus showed a more colourful plumage than birds parasitized by more than one parasite genus. In spite of the temporal separation between the moult and the reproductive season (when we took colour measurements), the relationship between parasite richness and carotenoid chroma could be expected. Birds being able to develop a conspicuous plumage during the moult could show a better health status in the next spring, especially because most of these blood parasite infections are chronic with birds suffering relapses while breeding (Valkiūnas

2005). Birds in poor health would be less efficient in acquiring carotenoid-rich food (Hill 1991, 1992; Senar et al. 2002) and consequently would exhibit a paler plumage. A previous experiment in the same study population showed that females medicated with primaquine did not vary in body condition during the breeding season, whereas body condition was reduced in control females during the same period, suggesting that blood parasites have negative effects on female condition (Merino et al. 2000). In addition, Tomás et al. (2007) showed that females treated with high doses of the antimalarial drug were able to increase their reproductive effort in comparison to control females and those treated with low doses of the drug. These results support the existence of harmful effects induced by parasitism, parasitized birds being in poor condition and therefore showing a paler colour.

Furthermore, according to the hypotheses that deposition of carotenoids into feathers is traded-off with their participation in physiological functions as immune-stimulators and antioxidants (Lozano 1994; Møller et al. 2000), parasitized birds would have less carotenoids available for plumage pigmentation. An alternative non-excluding mechanism would be that carotenoid-based traits are signalling levels of other non-pigment antioxidants that might protect carotenoids from free radical attacks and make them available for sexual advertisements (Hartley and Kennedy 2004). Additionally, apart from the passive "protection mechanism" (Hartley and Kennedy 2004), the allocation of colourless antioxidants to sexual signalling may promote an active mechanism to increase the amount of pigments (Pérez et al. 2008). Another hypothesis suggests a trade-off between lipids being used for energy generation or for absorption and/or transportation of carotenoids (Fitze et al. 2007). Whatever the mechanism implied, it appears that carotenoid-based plumage colouration signals health status in our blue tit population, as multiple infections negatively affect the showiness of the yellow breast plumage.

Negative effects of multiple infections by blood parasites in birds have been also found in other studies (Davidar and Morton 2006; Arriero and Møller 2008; Marzal et al. 2008). This could be due to additive effects of infection by different blood parasite genera. Another possibility is that infection by a second genus could induce competition for resources between different parasites (Frank 1996) or activation of the immune response (Read and Taylor 2000), which would induce parasites to become more virulent than if they infected hosts separately (Frank 1996). It is assumed that parasites which grow more rapidly inflict more damage on their hosts and are competitively superior to less harmful parasites (see de Roode et al. 2005). Thus, if parasite competition is occurring, higher *Haemoproteus* infection intensity would be expected in birds infected by several blood parasites. In fact, we can expect that the intensity of *Haemoproteus* increases with the number of parasite genera infecting the same bird. However, there were not significant differences in the intensity of infection by *Haemoproteus* among birds infected by different numbers of parasite genera in our blue tit population. Therefore, competition between *Haemoproteus* and other parasites apparently does not exist or at least it is not reflected in an increase in the intensity of *Haemoproteus* infection. Nevertheless, further studies to explore the interactions between parasites inside a common host and their effects are needed.

On the other hand, we found an inter-annual variation in the effect of multiple infections on carotenoid chroma. In 2007, birds parasitized by two and three blood parasite genera were significantly more colourful than birds infected by two and three blood parasite genera in 2004. Carotenoids must be acquired through the diet (Olson and Owens 1998), caterpillars being the major source of these pigments for blue tits (Slagsvold and Lifjeld 1985). Thus, a year may be "favourable" for birds because there is a higher abundance of food (greater carotenoid availability) and/or a lower abundance of parasites, and consequently, more carotenoids would be available to be deposited into birds' feathers. Hence, it was not surprising to find inter-annual variation in the carotenoid chroma of birds, those of 2007 being more colourful than those of 2004. In this respect, the prevalence of blood parasites in 2007 was lower than in 2004 although *Haemoproteus* infection intensity was higher in 2007. Likewise, Hõrak et al. (2000) showed that great tit nestlings which grew up in a "bad" year had paler yellow breasts than nestlings which grew up in a "good" year. Different environmental conditions can also be found between habitats, explaining a large part of the variation in the colour of birds from habitats of different quality. Blue tits inhabiting structurally complex forests obtain more caterpillars (Ferns and Hinsley 2008), and consequently present higher chroma values than blue tits from poor quality forests (Arriero and Fargallo 2006). Our results suggest that birds of 2007 infected by two or three blood parasite genera had higher carotenoid availability for pigmentation in late 2006 (when they moulted their plumage) than birds of 2004 infected by two or three blood parasite genera in late 2003. However, birds infected by four or five blood parasite genera presented similar values of carotenoid chroma in both years, suggesting that highly parasitized birds are the palest birds independently of favourability of environmental conditions. Our main results were still significant when we only analysed parasite data based on PCR, supporting the association between carotenoid chroma and blood parasite richness and HSP70 levels. However, we can not rule out completely the possibility that differences between results using the expanded (PCR plus microscopic data) or the reduced sample (only PCR data) were due to the presence of false negatives and not only to reduction in sample size.

In addition, we did not find any relationship between *Haemoproteus* infection intensity and carotenoid-based colour of the breast, suggesting that blood parasite richness per se and not *Haemoproteus* intensity influenced carotenoid-based colour in our population. Whereas Merilä et al. (1999) found a negative relationship between *Haemoproteus* infection intensity and plumage yellowness of male greenfinches, *Carduelis chloris*, other authors did not find this relationship (Seutin 1994; Dufva and Allander 1995). These contradictory results may be due to the use of different methods to quantify haematozoan infections, as suggested by Clayton (1991), as well as differences in the colour variables used. On the other hand, we found a positive relationship between HSP60 levels and *Haemoproteus*. This is not surprising because in an experiment consisting of a reduction in the intensity of infection by *Haemoproteus* and the prevalence of infection by *Leucocytozoon* in female blue tits, Tomás et al. (2005) found that control females had a higher final level of HSP60 than medicated ones. Also, other studies on birds suggested a role of this protein in responses to parasitism (Merino et al. 1998; Merino et al. 2002; Arriero et al. 2008). Parasites may cause increases in HSP60 by at least three different, non mutually exclusive mechanisms: it could be a response to the fever occasioned by the infection, i.e. HSP60 could be increased in response to a heat shock (Garbe 1992); the increase in HSP60 could be due to a pathogen-induced necrosis (Moseley 2000); and secretion/excretion products from parasites could induce protein expression (Martínez et al. 1999b). Our result adds support to the hypothesis that parasites are the stressors that cause the increase in HSP60 values in blue tits. However, a relationship does not seem to exist between parasitism and HSP70 levels (Merino et al. 2002; Tomás et al. 2005), suggesting that this protein responds in a different manner, or in response to different stressors, than HSP60. More studies are needed to clarify these associations.

Sex and age differences of carotenoid-based colouration of blue tits have been reported in some populations (Figuerola et al. 1999; Hidalgo-García 2006) but not in others (Ferns and Hinsley 2008). In this study, adult birds of both sexes showed similar values of carotenoid chroma, whereas young males were significantly more colourful than young females. Additionally, we did not find age differences in the colour of males but we did in the colour of females, young females being paler than adult females. Therefore, it seems that blue tits are more or less dimorphic in the yellow plumage of the breast depending on the population considered. This may be due to the partial dependence of carotenoid-based plumage colour on environmental factors (Hadfield and Owens 2006) such as habitat and population density.

We found a relationship of primaquine treatment with carotenoid chroma, which was especially strong in males. Untreated birds showed significantly higher values of carotenoid chroma than treated birds. We treated birds with primaquine months after the deposition of carotenoids in feathers during the moult, so we did not expect a direct effect of this drug on birds' colours. It could be that this was a spurious effect, i.e. the colour of untreated birds was more intense prior to treatment; however, another possibility could be that higher provisioning rates of treated birds (Tomás et al. 2007) induced a higher abrasion of birds' plumages due to rubbing against nest box entrance or meant less time was devoted to feather maintenance activities resulting in paler colours (McGraw and Hill 2004). Studies taking into account colouration before treatment are needed to clarify the effect of primaquine on carotenoid-based plumage colour.

Since the carotenoid chroma of plumage reflected to a certain degree the blood parasite richness within a host, we could also expect it to reflect body condition, and immunoglobulin and HSP levels. Although Senar et al. (2003) found that carotenoid-based plumage colour of the breast of the great tit *Parus major* was correlated with the nutritional condition, as estimated by the rate of tail growth (see also Senar et al. 2008), and several authors have reported positive correlations between carotenoid-based colours and immune function (Dufva and Allander 1995; McGraw and Ardia 2003; Alonso-Álvarez et al. 2004), we could not find any association between carotenoid chroma and immunoglobulin levels or body condition. However, birds with lower carotenoid concentrations in feathers had higher HSP70 levels. These proteins prevent cellular homeostasis alteration and are involved in many different functions like protein synthesis, folding and transport, as well as in degradation of misfolded, non-functional proteins (Morimoto 1991). These proteins are implicated in responses to a wide array of stressors including blood parasites (Merino et al. 1998; Tomás et al. 2005). As previously reported (Merino et al. 2002; Tomás et al. 2005), we did not find *Haemoproteus* infection intensity significantly associated with levels of HSP70. However, the relationship between HSP70 and plumage colour may be in part related to an infection-associated stress. The fact that blood parasite infections are chronic and maintained in birds for several months and even years with periods of relapses (Valkiūnas 2005) may help to understand the existence of these associations between long-term stress indicators such as HSPs levels and plumage colours. On the other hand, this association may be due to the function of carotenoids as antioxidants in the organism (Lozano 1994; Møller et al. 2000) since HSPs respond to oxidant compounds as well (Martínez et al. 1999a). Therefore, more colourful birds would have more carotenoids available for this antioxidant function, fewer oxidant compounds and consequently lower HSP70 levels. As discussed above, HSP70 seems to respond in a different manner, or in response to different stressors, than HSP60.

Overall, based on the full data set, carotenoid-based plumage colouration seems to be a long-term indicator of health status in this population of blue tits. This study reveals negative effects of multiple infections by blood parasites on carotenoid-based plumage colour. Further experiments are needed to better understand the effects of multiple parasite infections on hosts and the mechanisms underlying carotenoid-based colouration.

Acknowledgments We thank Javier Donés (Director of Montes de Valsaín) for permission to work in the study area. The Junta de Castilla y León authorized the ringing and handling of birds. This study was funded by projects BOS2003-05724 and CGL2006-14129-C02-01 of the Ministerio de Educación y Ciencia (to S. M.) and CGL2007-61251 (to J. Moreno). The authors thank Sonia Aracil for their help in the lab. S. d. C., J.-M. P. and R. R. d. C. are supported by grants from the Comunidad de Madrid, El Ventorrillo-CSIC and JAE-CSIC respectively. J. Morales is supported by a postdoctoral grant from the Ministerio de Ciencia y Tecnología. E. L. was supported by a FPU grant from MEC. G. T. was supported at different stages by a FPI grant from the Comunidad de Madrid, an I3P postdoctoral contract from CSIC and a postdoctoral grant from UNAM. J.-R. A. is not supported by any grant. This study is a contribution to the research developed at El Ventorrillo Field station and complies with current Spanish laws.

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