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ORIGINAL PAPER

Carotenoid-based bill and eye ring coloration as honest signals of condition: an experimental test in the red-legged partridge (*Alectoris rufa*)

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Abstract Carotenoid pigments cannot be synthesized by vertebrates but must be ingested through the diet. As they seem to be a limited resource, carotenoid-based ornaments are particularly interesting as possible honest signals of individual quality, in particular of foraging efficiency and nutritional status. Some studies have demonstrated the condition dependence of carotenoid-based plumage in birds. However, many other carotenoid-pigmented bare parts (i.e. skin, caruncles, bills, cere, and tarsi) are present in birds but, in comparison with plumage, little is known about these traits as indicators of individual quality. Here, we show that the eye ring pigmentation and bill redness of the red-legged partridge (*Alectoris rufa*) are positively associated to body condition and recent changes in body mass. Also, we found a negative relationship between these two traits and heterophil-to-lymphocyte ratio, an indicator of physiological stress (the relationship with bill redness being significant only for males). In an experiment, we found that after a period of reduction in food intake (with the consequent loss of body mass), food-restricted birds showed lower eye ring pigmentation than ad-libitum-fed birds. Therefore, different ornaments seem to reflect changes in body condition but at different speeds or intensities (eye ring, a fleshy ornament, appears to respond more rapidly to changes in the nutritional status than a keratinized structure as the bill). These results indicate that carotenoid-based ornaments are condition-dependent traits in the red-legged partridge, being therefore susceptible to be employed as honest signals of quality in sexual selection.

Keywords Body condition · Carotenoids · Galliforms · Honest signaling · Ornaments · Sexual selection

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Introduction

Carotenoid pigments determine the red, orange, and yellow colors of many secondary sexual traits. Animals cannot synthesize carotenoids de novo but must ingest them with their food (Olson and Owens 1998). It has been proposed that carotenoid pigments serve as reliable signals of condition because their availability in the environment may be limited, and thus only good foragers would be able to accrue the pigments needed for maximum ornament expression (Endler 1983; Kodric-Brown 1985; Hill 1990). If energy-rich food items are also those with higher concentration of carotenoids, good foragers will obtain both higher levels of carotenoids and other nutrients, establishing a link between nutritional status and expression of carotenoid-based ornament. Alternatively, the most energy-rich foods may not be those containing the higher quantities of carotenoids. In that case, again only good foragers (those whose energetic demands are fulfilled) will be able to forage for carotenoid-rich (but energetically poor) food items, thus maintaining the condition–ornament expression link in a similar way. However, despite the widely held view that carotenoids may signal condition,

few studies relating foraging success or efficiency and carotenoid-based ornaments in birds have been published (e.g., Hill and Montgomerie 1994; Hill 2000; Casagrande et al. 2006).

Apart from condition and foraging ability, carotenoid-dependent signals may be honest indicators of the health status and parasite infection of individuals (Lozano 1994; Møller et al. 2000) or may also signal their capacity to prevent such infections (Hill and Farmer 2005; Dawson and Bortolotti 2006; Mougeot 2008). Furthermore, carotenoids play important roles in immunoregulation and immunostimulation, lymphocyte proliferation, and free radical scavenging (review in Møller et al. 2000). Hence, a tradeoff might be expected between investing carotenoids in self-maintenance versus ornament coloration because individuals that are forced to fight infections would have fewer carotenoids available for signal expression (Lozano 1994; Olson and Owens 1998; Møller et al. 2000).

Most of the published works linking condition and carotenoid coloration in birds have been performed in a few well-known model species that show carotenoids in their plumage (e.g., house finches (*Carpodacus mexicanus*): Hill and Montgomerie 1994; Hill 2000; or American goldfinches (*Carduelis tristis*): McGraw et al. 2005). However, although feathers could indicate physical condition at the time of molt, feathers cannot reflect short-term changes in physiological condition because they are nonliving structures. In contrast, other parts of birds (i.e., skin, caruncles, bills, cere, and tarsi) may be brightly colored as a result of carotenoid pigmentation but often have the potential to change either color or shape rapidly. Besides the fact that carotenoid pigmentation is more common in bare parts than in plumages (Olson and Owens 2005), the biological role and functional basis of these structures remain relatively understudied. In recent years, several experimental studies have shown that carotenoid-pigmented bare part (bills, combs) coloration may reflect current health status in some species (Faivre et al. 2003; McGraw and Ardia 2003; Peters et al. 2004; Alonso-Alvarez et al. 2004; Martínez-Padilla et al. 2007; Mougeot et al. 2007a, b). Although there is some correlational evidence about these traits as indicators of body condition (e.g., Casagrande et al. 2006; Birkhead et al. 1998; Mougeot et al. 2007a, b), experimental studies are scarce (Birkhead et al. 1998). In fact, this is especially true for brightly colored periocular rings and eye lores (eye rings hereafter). Besides being among the most widespread ornaments in birds, few studies have tested their validity as signals of quality (e.g., Blount et al. 2002; Kristiansen et al. 2006). In this sense, experimental studies in captivity where food access can be manipulated while other potentially confounding factors are controlled for are a good way to assess unambiguously the condition dependence of these ornaments.

In this paper, we experimentally assess whether bill and eye ring coloration are condition-dependent traits in the red-legged partridge (*Alectoris rufa*), a primarily monogamous, small-sized galliform (Cramp and Simmons 1980). In this species, both males and females show red coloration in the eye rings, bill, and tarsi, color being more intense in males than females (Villafuerte and Negro 1998, Pérez-Rodríguez 2008). Tissue samples from the red-colored traits, the ornaments, have been analyzed by high-performance liquid chromatography, and this analysis confirms that the red coloration under study is due to carotenoid pigments (R. Mateo, unpublished). Blood is not likely to contribute to red coloration of these traits as the color perceived is expressed in the epidermis (in the case of the eye ring) or in keratinized layers of the beak, externally to the living (irrigated) core of that structure. We analyzed the relationship between body condition, carotenoid-pigmented traits (eye ring and bill), and circulating levels of carotenoids in this species. Furthermore, because immune status may affect carotenoid-dependent ornaments, we predicted a negative relationship between two indices of immune system activation (total leukocyte counts and heterophil-to-lymphocyte ratio) and carotenoid-based ornamentation and blood carotenoid levels. In addition, we assessed the effect of experimentally reduced body condition on carotenoid-based coloration in this species. If carotenoid-based coloration is a condition-dependent trait in this species, then we would expect individuals with limited access to food to show lower eye ring and bill coloration than ad-libitum-fed birds. Finally, as males and females may differ in their signaling efforts (i.e., stronger sexual selection towards honest signaling may be exerted on males), we compared the effect of the experiment between sexes, and we expected males to meet our predictions better than females.

Materials and methods

Research protocol

The experiment was performed in the "Dehesa de Galiana" experimental facilities of the Instituto de Investigación de Recursos Cinegéticos (Ciudad Real, central Spain), during the breeding season (March–April) of 2003. The partridges used in the experiment were incubator-hatched the previous year from eggs coming from 70 different pairs. Birds were kept from December to February in two communal unisexual pens (8×10 m) and fed with a mixture of commercial pelleted food (20% protein, 4.5% fat, 3.7% cellulose; carotenoid content=4.4 μg/g) and wheat. Opaque walls visually isolated the two pens. In February, 42 males and 32 females were individually housed in cages (1×0.5×0.4 m) whose floor was made of wire mesh, at a height of 1 m over

the ground. The cages were visually isolated from each other, at ambient temperature and natural photoperiod, and received sunlight through the top. At the time of individual isolation, all birds were weighted with a Pesola spring balance (to the nearest 5 g) and medicated against coccidia by adding sulfaquinoxaline, a coccidiostatic of common use in partridge farms, to drinking water (1 ml/l during 1 week). The effectiveness of coccidiostatic treatment was confirmed by fecal analyses throughout the study period. Elevated cages where birds have no contact with the ground are considered the best system to maintain low levels of infection by coccidia and are routinely used in commercial farms. By controlling any possible infection by coccidia, we avoided the possibility that our experimental food restriction treatment (see below) had concomitant effects of these parasites that could be difficult to disentangle from the effect of body condition per se.

During the individual isolation period, caged birds were fed with only the pelleted food mentioned above and water was provided ad libitum. At this time, individuals were randomly assigned either to an experimentally food-restricted group (23 males and 17 females) or to a control group fed ad libitum (19 males and 15 females). The location of experimental cages was also randomized. The daily food intake of each partridge from the food-restricted group was calculated before the experiment during the first 2 weeks of March by adding a known amount of food and recovering the unconsumed portion in the feeder 24 h later. Measurements of daily consumption during those 15 days were repeatable (r=0.71, $F_{39,\ 160}$=13.23, P<0.001). Therefore, the average was considered to be an accurate estimate of the daily food intake of each individual. Female birds included in this study did not lay eggs during that breeding season, the rule for isolated 1-year-old females.

Just before the food restriction experiment started (in March, approximately 1 month and a half after the birds were individually housed in cages), all birds were weighted again and left tarsus length (to the nearest 0.01 mm), body length, and tail length (both to the nearest millimeter) were also measured. Tail damage is quite common in farm-reared birds, so its length was discounted from body length measurement. High-resolution (2,272×1,704 pixels) digital photographs of the left side of the head of each bird were collected under fluorescent light illumination and against a white standard background. A standard gray-color chip was placed close to the head of the bird in all pictures. Furthermore, we took a 400-μl blood sample from the brachial vein using a heparinized syringe. Although plasma carotenoids do not show significant variations during daytime (Pérez-Rodríguez et al. 2007), all blood samples and measurements were obtained approximately at the same hour of the day (between 10:00 and 12:00 hours) in order to avoid any bias due to sampling time in any of the parameters studied. Blood samples were kept cold (4°C) and centrifuged within 8 h, and plasma was stored at −20°C until analysis. Also, a drop of blood was smeared on individually marked microscope slides, air-dried, and fixed in ethanol so that we could later count white blood cells.

Each bird from the food-restricted group was provided with 70% of its daily food intake during the first week and with 50% during the rest of the experiment. Body mass was measured every other day during the first 2 weeks of the experiment and daily afterwards. Food restriction was maintained until 1 day after each experimental bird had lost 15% of its initial body mass. Then, body mass was again measured, a second photograph of the head was taken and another set of blood samples were collected. When a bird of the food-restricted group was sampled after the treatment, a randomly selected bird of the same sex from the control group was also sampled on the same way, avoiding differences in sampling date between groups. After the experiment, all birds were fed ad libitum and returned to the original communal pens where they recovered initial weights. The imposed weight loss was similar to that of other previously published studies of food restriction (e.g., Totzke et al. 1999). Although weight loss was significant, final body mass was not lower than that found in some birds reared in captivity (L. Pérez-Rodríguez, personal observations) or in the wild (F. Buenestado, personal communication). Also, faster weight losses than those we experimentally induced have been previously recorded in unmanipulated partridges that suffered any physiological (e.g., parasite infection) or social (i.e., being placed in a new unknown pen) stressful situation. Therefore, we are confident that our experimental treatment was within the physiological range of the species, replicating situations of loss of body condition that may be naturally found.

Color measurements

There exists an obvious variability between birds in the relative proportion of bare skin around the eye covered by red pigmentation or showing the white skin underneath. Therefore, for each picture, we calculated the percentage of pixels of the eye lore skin pigmented by carotenoids (eye ring pigmentation hereafter) using Adobe Photoshop v 7.0. Measurements of eye ring pigmentation were highly repeatable (r=0.97, $F_{19,\ 20}$=74.3, P<0.001)

We also calculated the red (R), green (G), and blue (B) components of the eye ring (only the pigmented portion of the eye ring was considered), nostril, upper mandible, and lower mandible separately. The same components were calculated for the gray reference. Following previous studies with this (Villafuerte and Negro 1998; Blas et al. 2006) and other carotenoid-pigmented species (Pike et al.

2007), the intensity of carotenoid-based red coloration (redness hereafter) was calculated as R divided by the average of R, G, and B. In order to control for any subtle variation in illumination between pictures, redness of the gray reference was entered as a covariate in all the analyses where ornament redness was the dependent variable. Redness measurements of the eye ring, nostril, and upper and lowers mandible were repeatable ($r>0.90$, $F_{19,\ 20}>20.1$, $P<0.001$, in all cases). We acknowledge that the range of our color measurements is less extended than the colors perceived by the birds, which possess receptors for UV light (Cuthill et al. 2000). However, despite this limitation, information obtained from digital pictures is still very useful as it reveals patterns and effects of biological meaning (e.g., Blas et al. 2006; Martínez-Padilla et al. 2007; Mougeot et al. 2007b; Pike et al. 2007).

Carotenoid analysis

Carotenoids in plasma were quantified by diluting 60 μl of plasma in acetone (1:10). The mixture was vortexed and centrifuged at 10,000 rpm for 10 min to precipitate the flocculent proteins. The supernatant was examined in a Shimadzu UV-1603 spectrophotometer and we determined the optical density at 446 nm, the wavelength of maximal absorbance for lutein (Mínguez-Mosquera 1993), the most abundant carotenoid in plasma, and commercial food of partridges. Finally, plasma carotenoid concentration (microgram per milliliter) was calculated using a standard curve of lutein (Sigma Chemicals). Measurements of plasma carotenoids showed high repeatability ($r=0.99$, $F_{19,\ 20}=602.2$, $P<0.001$)

Blood cell counts

For leukocyte counts, blood smears were stained with Quick Panoptic (QCA S.A., Amposta, Spain). Ten fields of homogeneous monolayer cell density of each blood smear were scanned under ×40 lens and the total number of white blood cells (WBC) was noted. The proportions of heterophils and lymphocytes (other cell types were less than 2% of total leukocytes) were assessed on the basis of examination of 100 leukocytes under ×100 lens under oil immersion. WBC count is a typical trait of inflammatory processes in response to microbial and macroparasite infections and may reflect current investment in immune defence (e.g., Møller 1998; Nunn et al. 2000). The heterophil-to-lymphocyte (H–L) ratio is widely used to estimate stress in poultry (Maxwell 1993) and also in wild birds (Birkhead et al. 1998; Totzke et al. 1999). A subset of 20 blood smears was scanned twice in order to assess repeatability of these two variables ($r=0.86$, $F_{19,\ 20}=13.4$, $P<0.001$ and $r=0.89$, $F_{19,\ 20}=17.9$, $P<0.001$, for WBC and H–L ratio, respectively). All blood smears were also scanned (80 fields under ×40 lens) for the presence of blood parasites, but results were negative in all cases. Absence of blood parasites in this sample has been later confirmed by genetic analyses (J.T. Garcia, unpublished data)

Statistical analyses

For simplification and adjustment of the analysis to the possible biological meaning of the colored structures considered, we separated color variables of the eye ring (eye ring pigmentation and eye ring redness) from those measured on the bill (nostril and upper and lower mandible redness), which are more keratinized structures. This separation is meaningful because eye rings are soft tissues and may therefore change color more rapidly and better reflect current physiological status of the individual. To evaluate overall bill redness, we conducted a principal component analysis (PCA) on bill color variables (nostril, upper mandible, and lower mandible). The first principal component ("bill redness," hereafter) explained 61% of variance, with nostril and upper and lower mandible redness all having positive loadings (0.73, 0.82, and 0.79, respectively). We thus used PC1 scores as an index of overall bill redness.

Tarsus and body length were pooled in another PCA, obtaining a first principal component ("body size," hereafter) that explained 71% of variance in these two variables. Standardized residuals of the regression of body mass over body size (obtained separately for each sex, given the sexual dimorphism of the species) were employed as a body condition index ("body condition," hereafter) in correlational analyses. These correlational analyses were performed on data from all birds before experimental treatment by means of general linear models (GLMs). Carotenoid-based ornamentation (eye ring pigmentation, eye ring redness, or bill redness) and plasma carotenoids were entered as dependent variables in four different models. Sex, plasma carotenoids (in the models for carotenoid-based ornamentation), body condition, WBC count, and H–L ratio were entered as fixed factors. When eye ring or bill redness were the dependent variables, redness of the grey standard reference was entered as a covariate in the models. It has been shown that carotenoid-based coloration in this species may reflect recent changes in body mass of the individual (Blas et al. 2006). Consequently, we also included the change in mass experienced by each bird between individual isolation in cages and the time when blood and digital photographs were collected (just before experimental food restriction, that is, approximately 1 month and a half later) as a fixed factor in the models (results did not differ when relative, instead of absolute, body mass changes were employed; data not shown). Furthermore, as

the relationship of these factors with plasma carotenoids and carotenoid-based coloration may differ between sexes, we also included the interaction between sex and each fixed factor in the initial models. Nonsignificant terms ($P>0.05$) were sequentially excluded from the model using a backward stepwise selection.

To test the effect of our experimental treatment on body mass, plasma carotenoids, and carotenoid-based ornamentation, we employed general linear mixed models (GLMMs). Sex, time (before or after food restriction), treatment (fed ad libitum or restricted), and their interactions were entered as fixed factors. Again, when eye ring redness or bill redness were the dependent variables, redness of the standard gray reference was entered as a covariate in the model in order to control for any subtle variation in illumination between pictures. The effect of experimental treatment on hematological variables is not shown here as it will be analyzed in a separate study.

Some variables required arc sine (eye ring pigmentation) or logarithmic (WBC counts and H–L ratio) transformations. After that, all variables were normally distributed (Kolmogorov–Smirnov tests). Analyses were performed with the statistical softwares SAS v 9.0 (GLMMs) and Statistica v 6.0 (GLMs).

Results

Carotenoid-based ornamentation, circulating carotenoids, body condition, and hematological variables before experimental treatment

Significant variables affecting eye ring pigmentation, eye ring redness, bill redness, and plasma carotenoids before experimental treatment are summarized in Table 1. The only variable analyzed that was significantly related to circulating carotenoids was the sex of the individual, males showing higher levels than females (Table 1). Eye ring pigmentation was positively and significantly related to circulating carotenoids, body mass increase during the first month of individual isolation in cages, and body condition at the time of color measurement (Fig. 1, Table 1). In contrast, a negative significant relationship with H–L ratio was found in both sexes (Table 1). When we analyzed the redness of the pigmented area of the eye ring, the only factor showing a significant effect was the sex of the individual, males showing redder eye rings than females (Fig. 1, Table 1). Bill redness was significantly and positively related to body mass increase during the first month of individual isolation and body condition (Fig. 1, Table 1). Bill redness was also sexually dimorphic, males showing redder bills than females (Fig. 1, Table 1). Furthermore, there was a significant interaction between H–L ratio and sex affecting bill redness. Post hoc analyses revealed that H–L ratio was negatively and significantly related to bill redness in males ($beta=-0.45$, $F_{1,\ 30}=7.79$, $P=0.009$). In contrast, these two variables were not significantly related in females ($beta=-0.001$, $F_{1,\ 20}=0.00$, $P=0.97$).

Daily food intake was measured for birds that were going to be included in the experimental food-restricted group. For that subsample of birds, average daily food intake did not differ between sexes ($F_{1,\ 38}=0.56$, $P=0.97$) and was not related to body size ($F_{1,\ 38}=0.75$, $P=0.39$), eye ring pigmentation ($F_{1,\ 32}=2.33$, $P=0.13$), eye ring redness ($F_{1,\ 34}=1.01$, $P=0.32$), or circulating carotenoids ($F_{1,\ 36}=0.00$, $P=0.95$; interactions were not significant either, all $P>0.23$). However, a significant positive relationship was found between bill redness and daily food intake ($F_{1,\ 34}=20.9$, $P<0.001$).

Table 1 Factors affecting plasma carotenoids, eye ring pigmentation, eye ring redness, and bill redness of red-legged partridges before experimental food restriction

Dependent variable	Factor	*F* value	*df*	*P* value
Plasma carotenoids	Sex	4.44	1, 55	0.039
Eye ring pigmentation	Body condition	6.36	1, 46	0.015
	Body mass change	7.61	1, 46	0.008
	Plasma carotenoids	4.98	1, 46	0.030
	H–L ratio	16.4	1, 46	<0.001
Eye ring redness[a]	Sex	10.5	1, 51	0.002
Bill redness[a]	Sex	4.44	1, 47	0.040
	Body condition	20.5	1, 47	<0.001
	Body mass changes	6.74	1, 47	0.012
	H–L ratio	0.77	1, 47	0.38
	Sex × H–L ratio	4.15	1, 47	0.039

[a] Redness of the gray reference as a covariate ($F_{1,\ 51}=1.71$, $P=0.19$ and $F_{1,\ 47}=5.75$, $P=0.02$ for eye ring and bill redness, respectively)

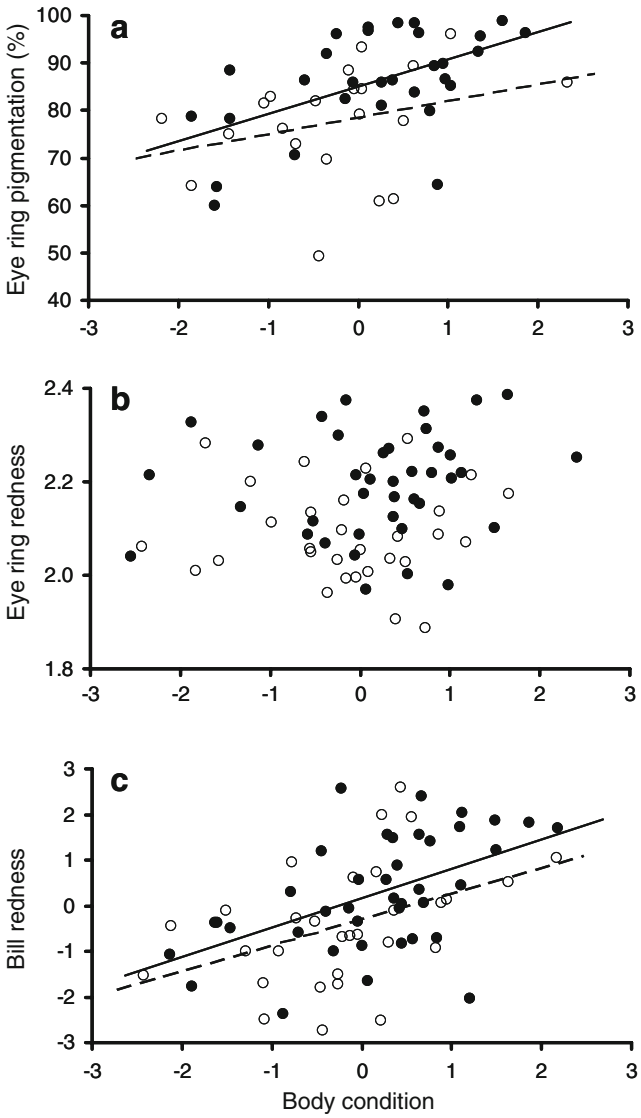

Fig. 1 Relationship between body condition (standardized residuals of the regression of body mass over body size) before experimental treatment and **a** eye ring pigmentation, **b** eye ring redness, and **c** bill redness. *Tendency lines* have been added only for those variables significantly related. In all cases, *closed circles* and *solid lines* correspond to males whereas *open circles* and *dashed lines* correspond to females

Effects of experimental food restriction on plasma carotenoids and carotenoid-based ornamentation

Before the food restriction experiment started, control and food-restricted birds did not differ significantly in any of the variables analyzed ($F_{1,\ 62–72}$=0.03–0.06, all P>0.80). We found a significant effect of treatment on body mass as revealed by the significant interaction between treatment (control or food-restricted) and sampling time (before or after food restriction; GLMM: $F_{1,\ 70}$=279.5, P=0.004), showing that our experiment was effective in creating differences in body condition.

The effect of experimental food restriction on studied variables is detailed on Table 2. Food restriction significantly affected eye ring pigmentation (Fig. 2, Table 2). Control birds increased eye ring pigmentation ($F_{1,\ 30.8}$= 17.8, P<0.001), whereas food-restricted individuals showed no change during the study ($F_{1,\ 37.7}$=0.36, P= 0.55; Fig. 2). The effect did not differ between sexes (no significant sex × time × treatment interaction, Table 2). In contrast, eye ring redness and bill redness were not affected by the experimental treatment but increased during the study in both sexes and groups (Fig. 2, Table 2). Circulating carotenoids were negatively affected by the experimental treatment, similarly so in both sexes (Fig. 2, Table 2), food-deprived individuals showing a decrease in plasma carotenoids of approximately 15% of their initial levels. In contrast, plasma carotenoids increased in control birds, although this change was not significant (time: $F_{1,\ 30.8}$= 3.21, P=0.08; sex × time interaction: $F_{1,\ 30.8}$=2.98, P= 0.09). Differences between sexes in the effect of the treatment were not significant either (Table 2).

Discussion

Our results support the initial hypothesis that carotenoid-based coloration honestly reflects body condition in the red-legged partridge. Correlational analyses showed that eye

Table 2 Effect of the experimental food restriction on plasma carotenoids, eye ring pigmentation, eye ring redness, and bill redness of red-legged partridges. Sex, sampling time (before or after), and treatment (fed ad libitum or restricted) are fixed factors. Individual was entered as a random variable

Factors	Plasma carotenoids			Eye ring pigmentation			Eye ring redness[a]			Bill redness[a]		
	F value	*df*	*P* value	*F* value	*df*	*P* value	*F* value	*df*	*P* value	*F* value	*df*	*P* value
Sex	7.93	1, 71.2	0.006	3.43	1, 68.9	0.07	30.1	1, 66.5	<0.001	1.22	1, 70.2	0.27
Time	1.22	1, 68.4	0.27	10.2	1, 63.4	0.002	21.4	1, 64.7	<0.001	21.4	1, 67.6	<0.001
Treatment	5.2	1, 71.2	0.02	0.5	1, 68.9	0.48	0.82	1, 66.5	0.36	0.00	1, 70.3	0.95
Time × treatment	16.5	1, 68.4	<0.001	13.9	1, 63.4	<0.001	0.53	1, 65.8	0.47	0.00	1, 68.3	0.98
Sex × time × treatment	1.37	3, 87.2	0.25	0.34	3, 87.9	0.79	1.06	3, 99.6	0.37	0.08	3, 95.7	0.97

[a] Redness of the gray reference as a covariate ($F_{1,\ 133}$=0.43, P=0.52 and $F_{1,\ 128}$=3.67, P=0.06 for eye ring and bill redness, respectively)

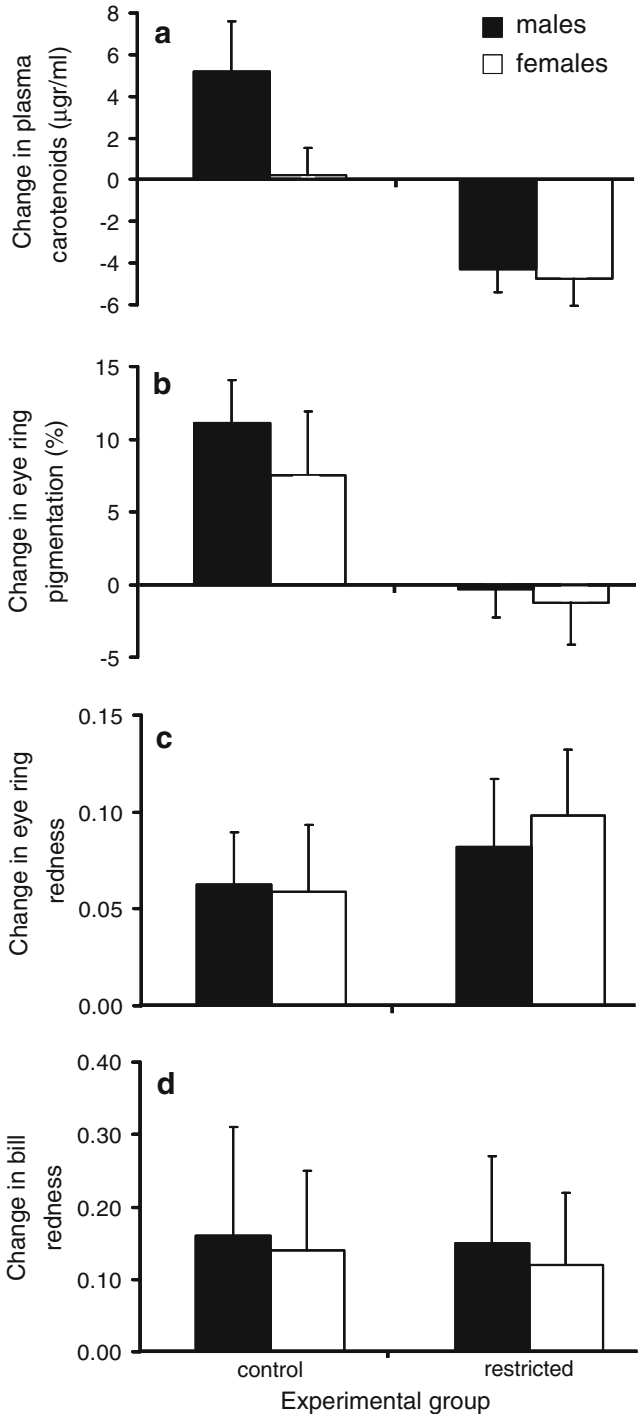

Fig. 2 Changes in **a** plasma carotenoids, **b** eye ring pigmentation, **c** eye ring redness, and **d** bill redness during the experiment in males (*black columns*) and females (*open columns*) fed ad libitum (control) or under food restriction. Values are mean ± SE of absolute changes

ring pigmentation and bill redness were significantly related to body condition and recent body mass changes in this species. Furthermore, experimental food restriction affected eye ring pigmentation, although no effect of the experimental treatment was evidenced for bill or eye ring redness.

Ornaments based on live tissues, in contrast to those expressed in the plumage, may change quickly according to current status to convey up-to-date information about the individual. For instance, carotenoid-based skin color has been found to change within 48 h in blue-footed booby (*Sula nebouxi*) as a response to food deprivation (Velando et al. 2006). Similarly, carotenoid-based color of red grouse (*Lagopus lagopus scoticus*) combs increased 18 days after intestinal nematode removal (Martínez-Padilla et al. 2007) and bill color of male blackbirds (*Turdus merula*) significantly decreased during the course of a 7-day-long experimental immune challenge (Faivre et al. 2003). The eye ring pigmentation of red-legged partridges of this experiment changed markedly in the 21.6±0.8 days during which experimental food restriction was performed. However, such a rapid response was not found in the case of bill and eye ring redness. Bill redness may be affected by long-term body condition (as revealed by our correlational results), while we failed to find any relationship between eye ring redness and body condition. Overall, the results of the correlational analyses and the experimental treatment suggest that each trait considered responds in different ways to changes in nutritional status. The eye ring is a fleshy structure whose pigmentation may be a more labile trait and probably the turnover of carotenoids on it takes place rapidly. In contrast to the eye ring, the bill is a keratinized structure and probably the turnover of carotenoids deposited on it takes place more slowly. Therefore, both traits (eye ring pigmentation and bill redness) may be linked to body condition but in different ways, eye ring reflecting short-term variations in nutritional status, whereas bill redness is a more stable trait that reflects body condition at longer term and requires more persistent stressful situations to change. Interestingly, our correlational data show that both eye ring pigmentation and bill redness reflected not only body condition but also changes in body mass occurred during the 1 month and a half period of individual isolation elapsed before the food restriction experiment (approximately twice the average duration of the food restriction experiment). Future studies where body condition is manipulated for a longer period of time than here are required to experimentally assess this hypothesis.

Eye and bill redness were unaffected by food restriction and increased along the experiment in a similar way in food-restricted and control birds. The same applies to eye ring pigmentation in control birds. All these trends are consistent with the seasonal variation in coloration already reported in this species (Pérez-Rodríguez 2008), in which carotenoid-based coloration reach maximum intensity approximately in April–May, by the end of mating season, which is also consistent with a role of these ornaments in sexual signaling (Pérez-Rodríguez 2008).

The most obvious explanation for the decrease in eye ring pigmentation in food-deprived birds is that our food

restriction experiment, apart from limiting the amount of nutrients available for birds, reduced the amount of carotenoids ingested. The extent of eye ring color before the experiment was related to circulating levels of carotenoids and our treatment induced a decrease in plasma carotenoids. However, although at the time of postrestriction sampling all food-deprived birds were ingesting 50% of their daily food intake (and hence 50% of carotenoids), their circulating levels of blood carotenoids decreased only by 15% with respect to prerestriction values. One possible explanation is that food-deprived birds partially compensated the reduction of carotenoids ingested by actively releasing them from body stores such as fat or liver (Negro et al. 2001a). Alternatively, carotenoids stored in body fat may have been released passively to the bloodstream when fat was mobilized as a result of the decrease in food intake. However, to date, it is unknown whether carotenoids stored in fat can be selectively mobilized when they are required by the bird (Negro et al. 2001a). Another possibility to explain the smaller than expected decrease in plasma carotenoid levels of food-deprived birds is that food-restricted birds compensated their lower carotenoid intake by increasing their absorption efficiency. However, the latter possibility seems unlikely given that carotenoid absorption is energetically costly (Hill 2000; McGraw et al. 2005) and therefore less feasible for food-restricted birds.

Food deprivation reduced the extent of the colored area in a soft tissue like the eye ring, but it did not affect the redness of the area that remained colored, that even increased along the experiment, a result somehow puzzling. A possible explanation is that, when carotenoid availability is low, birds would favor maintenance of color intensity while reducing pigmented area. This result also suggests that this soft tissue needs constant deposition of carotenoids to maintain or increase the area colored but that the intensity of color would be more dependent on other factors than carotenoid or food availability per se. Little is known about the biochemical processes affecting carotenoids between ingestion and deposition in colored structures and how these processes may affect color intensity (McGraw 2006). Our ongoing research on biochemical composition of the red tissues of this species indicates that, although astaxanthin is the main carotenoid present in these tissues, color is provided by a complex array of derived forms of carotenoid molecules experiencing biochemical processes such as oxidation and esterification (R Mateo, unpublished data). Perhaps changes in color intensity in our experiment were more dependent on biochemical processes affecting molecular structure of pigments already available before food deprivation, and this process should be little sensitive to food availability. This is a crucial aspect of research on carotenoids that deserves urgent attention in the near future.

Other factors may also contribute to the condition dependence of eye ring coloration in red-legged partridges. For instance, before being deposited in the ornament, ingested carotenoids suffer energetically demanding metabolic transformations that may not be afforded in the same way for birds in poor and good condition (Hill 2000; McGraw et al. 2005). Furthermore, at least in males, lower body condition is associated with a decrease in testosterone levels (Pérez-Rodríguez et al. 2006), which has recently been shown to be related to carotenoid absorption (McGraw et al. 2006; Blas et al. 2006), thus providing an extra way by which carotenoid-based coloration might be limited in males.

We also found that eye ring pigmentation and bill redness (the latter factor, only in the case of males), apart from being condition dependent, were negatively related to H–L ratio. Similar negative relationships between carotenoid-based coloration and H–L ratios have been found, for example, in greenfinches (*Carduelis chloris*; Saks et al. 2003) and zebra finches (*Taeniopygia guttata*; Birkhead et al. 1998). However, coloration was not related to WBC count in the bloodstream in either sex. Because heterophils are involved in the acute inflammatory response, their concentration in bloodstream (relative to lymphocyte number) is known to rise in response to microbial pathogens and high corticosterone levels; H–L ratio is a widely employed index of stress in birds (Maxwell 1993; Birkhead et al. 1998; Totzke et al. 1999). Thus, our results suggest that birds with higher color scores were in better general health status and were suffering lower stress than birds with low scores.

Our finding that overall carotenoid-based ornamentation was more intense in males than females is also consistent with the results of previous studies (Villafuerte and Negro 1998; Pérez-Rodríguez 2008). The sexual dimorphism in plasma carotenoids detected (consistent with the results of Negro et al. 2001b; Pérez-Rodríguez 2008) is the most plausible and parsimonious proximate cause. Differences in allocation priorities between sexes or the positive effect of testosterone on carotenoid absorption capacity in males (McGraw et al. 2006; Blas et al. 2006) may also explain the sexual dimorphism in plasma carotenoids and carotenoid-based ornamentation. Interestingly, although daily food intake was not related to plasma carotenoids and did not differ between sexes—which is consistent with results on zebra finches (McGraw et al. 2003)—daily food intake was significantly and positively related to bill redness irrespective of sex. This result supports the idea that this ornamental trait may indicate foraging efficiency in this species.

Except in the case of the negative relationship between bill redness and H–L ratio (that was nonsignificant for females), none of the results presented here differed between sexes. This suggests that the signaling potential

of these traits would be valid for males and females and that sexual selection may be acting in the same direction in both sexes, although the subtle sexual dimorphism in coloration suggests that the intensity of selection is stronger in males (Andersson 1994). Interestingly, recent data show that wild red-legged partridges mate assortatively with respect to carotenoid-based coloration (F. Mougeot et al., unpublished data), which may suggest that both sexes benefit from selecting their mates according to these traits. Therefore, our results suggest that eye ring and bill carotenoid-based coloration might have evolved as cues for assessing the quality of potential mates in the red-legged partridge. By selecting their mates according to these traits, individuals would benefit from mating with birds of superior foraging efficiency and better body condition.

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