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Carotenoid-based coloration predicts resistance to oxidative damage during immune challenge

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SUMMARY

Many animal ornaments may have evolved as signals advertising the quality of the bearer. The honesty of the information content of these signals would rely on the costs associated with their expression, these being relatively greater for low-quality than for high-quality individuals. Given the physiological functions of carotenoids, carotenoid-based ornaments could indicate individual immunocompetence, and possibly the ability to mount an immune response at a lower cost. We evaluated whether the red carotenoid-based coloration of male red-legged partridges (*Alectoris rufa*) predicts the capacity of the individual to counteract the oxidative stress generated by a cell-mediated immune response. Individuals were subcutaneously injected with phytohaemagglutinin (PHA) or phosphate buffer solution (PBS) as a control. We found that eye ring pigmentation predicted the change in the amount of peroxidized lipids (TBARS) in blood after the PHA-induced inflammatory challenge. The degree of pigmentation of this carotenoid-based ornament was also negatively related to individual changes in γ-glutamyl transferase (GGT), another biomarker of oxidative stress involved in antioxidant metabolism (i.e. glutathione recycling). However, changes in circulating carotenoids did not significantly explain changes in lipid peroxidation or GGT levels, suggesting that the higher resistance to oxidative stress of those individuals with more pigmented eye rings was not directly mediated by their greater circulating levels of carotenoids. Our results indicate that carotenoid-based coloration can predict not only immune responsiveness (more coloured males mount greater responses) but also an individual's ability to counter the oxidative stress generated during immune challenge (more coloured males experience less oxidative damage when mounting an immune response).

Key words: carotenoids, immune response, lipid peroxidation, ornaments, oxidative stress.

INTRODUCTION

Animal ornaments may have evolved as honest signals of individual quality for opponents or potential mates (Andersson, 1994). The honesty of these signals relies on the costs associated with their expression or maintenance, which should be relatively lower for high-quality than for low-quality individuals (Zahavi and Zahavi, 1997). Many experiments have evidenced the production costs of displaying elaborate ornaments, but the relative differences in such costs depending on individual quality have often been overlooked. In fact, much emphasis has been put on treatment level effects to the detriment of analyses of individual responses to treatments (but see Loyau et al., 2005; Dawson and Bortolotti, 2006; Mougeot et al., 2009a; Bortolotti et al., 2009).

Carotenoid-based ornaments are common, well-studied social signals (Hill and McGraw, 2006). Animals must acquire carotenoids through diet, so these traits were initially considered indicators of condition and foraging ability (Endler, 1980). In addition, carotenoids may have key physiological roles, acting as immunostimulants or antioxidants (Lozano, 1994; Olson and Owens, 1998). As the amount of available carotenoids is often limited, individuals face an allocation trade-off between self-maintenance needs and ornamental pigmentation (Olson and Owens, 1998). However, the role of carotenoids as significant antioxidants is still controversial (Hartley and Kennedy, 2004; Costantini and Møller, 2008; Pérez-Rodríguez, 2009). Individuals displaying brighter carotenoid-based ornaments usually mount greater responses to immune challenges (e.g. Blount et al., 2003; Pérez-Rodríguez et al., 2008), indicating that high-quality individuals are able to both allocate more carotenoids to signalling and raise greater responses. However, the immune response may be associated with increased oxidative stress (Costantini and Møller, 2009; Sorci and Faivre, 2009; Mougeot et al., 2010). This is because free radical production increases during an immune response and helps to counter invading pathogens (Halliwell and Gutteridge, 2007). The toxicity of free radicals is not restricted to the pathogen and their overproduction can damage host tissues, which may constitute a significant physiological cost associated with the immune response (Costantini and Møller, 2009; Sorci and Faivre, 2009). This raises the question of whether more ornamented birds suffer relatively lower physiological costs (in terms of oxidative damage) when facing an immune challenge.

Living tissues pigmented by carotenoids (beaks, skin, caruncules) are likely to reflect current physiological conditions better than 'dead' structures (e.g. feathers), being considered as dynamic traits (e.g. Faivre et al., 2003; Velando et al., 2006; Pérez-Rodríguez, 2008; Biard et al., 2009). However, the carotenoid turnover may differ between living tissues, which may result in some carotenoid-based traits indicating immediate quality whereas others may indicate

medium- or long-term quality (Pérez-Rodríguez and Viñuela, 2008; Pérez-Rodríguez et al., 2008; Mougeot et al., 2009b; Mougeot et al., 2010; Biard et al., 2009).

The carotenoid-based ornamentation of the red-legged partridge (*Alectoris rufa* Linnaeus) is an honest predictor of cell-mediated immunocompetence in males (Pérez-Rodríguez et al., 2008; Mougeot et al., 2009b). We tested here whether the carotenoid-based ornamentation (beaks and eye rings) also indicates an individual's capacity to counteract the oxidative stress generated during an immune response. Some birds were subcutaneously injected with phytohaemagglutinin (PHA) while other birds were injected with phosphate buffer solution (PBS) as a control. We expected the more pigmented individuals to suffer less oxidative damage after the challenge than paler individuals. Oxidative stress was evaluated by means of two different indices: lipid peroxidation and activity of the enzyme γ-glutamyl transferase (GGT). Lipid peroxidation (measured as the concentration of thiobarbituric acid reactive substances, TBARS) is a widespread and accepted index of oxidative damage (Monaghan et al., 2009). In red-legged partridges, lipid oxidative damage estimated using this technique closely tracked changes in oxidative stress associated with experimental testosterone increases (Alonso-Alvarez et al., 2008) or with ageing (Alonso-Alvarez et al., 2009; Alonso-Alvarez et al., 2010), and was associated with decreased hatching success (Alonso-Alvarez et al., 2010). GGT activity has recently been proposed as a good biomarker of oxidative stress (Lee et al., 2004) because this enzyme is involved in the recycling of a key endogenous antioxidant (i.e. glutathione) (Meister, 1983; Anderson, 1998; Wu et al., 2009). High GGT activity has been related to high levels of oxidative stress in humans and rodents (e.g. Shi et al., 1993; Lim et al., 2004; Yamada et al., 2006; Simao et al., 2008). In our species, the red-legged partridge, circulating carotenoids are positively related to carotenoid-based ornamentation (more coloured males have more circulating carotenoids) and rapidly decrease when the birds mount an immune response after PHA injection (Pérez-Rodríguez et al., 2008). Therefore, individuals with greater carotenoid-based ornamentation could suffer less oxidative damage because they have more circulating carotenoids, if carotenoids help counter oxidative stress (see Hartley and Kennedy, 2004; Costantini and Møller, 2008; Pérez-Rodríguez, 2009). We tested this possibility by exploring whether the initial circulating carotenoid levels or the changes in carotenoid levels before and after PHA injection explained variation in the amount of oxidative stress generated during an immune response.

MATERIALS AND METHODS

General procedure

We conducted the experiment on 40, 6 month old male partridges, which were hatched and reared in captivity and housed individually in outdoor cages. During the experiment, birds were fed *ad libitum* with commercial food pellets containing 5.26 μg of carotenoids (96% lutein) per gram of food. Although the carotenoid content of the diet of wild red-legged partridges is unknown, this concentration in food is routinely used and recommended for partridges reared in captivity (Blas et al., 2006; Pérez-Rodríguez et al., 2007; Pérez-Rodríguez, 2008). On December 11th, after 2 weeks of adaptation to cages, a blood sample (0.8 ml) was taken from the brachial vein of the right wing of each male. Blood was stored at 4°C and centrifuged within 4 h of collection (no signs of haemolysis were detected during this time interval). Blood was centrifuged at 11,000 ***g*** in order to separate the plasma and blood cell fraction (i.e. pellet), and both were immediately stored at –80°C. We also took a digital photograph of the left side of the head close to a grey standard reference, to assess beak and eye ring redness and the percentage of the eye ring surface pigmented by carotenoids. All pictures were taken under fluorescent light and against a grey standard background. The distance from the camera (Nikon Coolpix 4500; Tokyo, Japan) to the bird was held constant (40 cm). A grey standard reference (Kodak Gray Scale, Kodak, Rochester, NY, USA) was placed next to the head of the bird in all pictures.

On December 14th, 23 males were injected subcutaneously in the left wing patagium with 0.5 mg of PHA (Sigma-Aldrich, St Louis, MO, USA; ref L-8754) suspended in 0.1 ml of PBS (PHA group) and 17 males were injected with the same volume of PBS only (control group). We took a new blood sample 24 h later from the right wing as described above. This concentration of PHA was chosen according to the body mass of the species and to previous PHA tests conducted on this species (e.g. Blas et al., 2006; Mougeot et al., 2009b). Our treatment illicited the expected cell-mediated immune response (average wing web swelling ± s.e.: 1.32±0.06 mm) (see Pérez-Rodríguez et al., 2008). During the experiment, changes in body mass did not differ between PHA-challenged and control individuals (Pérez-Rodríguez et al., 2008). We thus had no evidence that immune challenges influenced body condition, or caused ataxia or abnormal behaviour. This was expected from this relatively harmless immune test routinely used in ecological studies on birds (Smits et al., 1999; Merino et al., 1999). Our research protocol was supervised by veterinary staff of the *Instituto de Investigación en Recursos Cinegéticos* and was performed according to Spanish laws.

Laboratory analyses

Lipid peroxidation was assessed by analysing TBARS in erythrocytes (Aust, 1985). The principle is based on the fact that most tissues contain a mixture of TBARS, including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. Samples were thawed and a small aliquot of each blood pellet was carefully pipetted, avoiding the surface layer. The exact mass of each aliquot was determined with a precision balance (±0.01 mg) and immediately diluted (1:10) and homogenized in a stock buffer (0.01 mol l^{-1} PBS and 0.02 mol l^{-1} EDTA), always working on ice to avoid oxidation. A 1 ml sample of the homogenate was mixed with 2 ml of a solution sensitive to TBARS (trichloroacetic acid 15%, HCl at 0.25 mol l^{-1} and thiobarbituric acid 0.375%) and 1 ml of 2% BHT (2,6-di-tert-butyl-4-methylphenol) in closed glass tubes. Tubes were then warmed for 30 min at 90°C and afterwards cooled with ice-cold water. The absorbance of the supernatant was then determined by spectrophotometry at 535 nm after centrifugation (2025 ***g***, 15 min). Concentrations of peroxidized lipids were determined by comparing absorbance data with those obtained from a curve produced with different malondialdehyde concentrations (i.e. end products of lipid peroxidation) (Aust, 1985) and expressed as nanomoles of malondialdehyde per gram of pellet analysed. TBARS measurements were significantly repeatable (R=0.76, P<0.001).

GGT activity (units l^{-1}) was determined in plasma samples using a spectrophotometer (A-25, Biosystems SA, Barcelona, Spain; http://www.biosystems-sa.com/welcome.htm), and commercial kits (ref. 11520) and certified controls (ref. 18005; Biosystems). Briefly, the principle of the test is based on the fact that GGT catalyses the transfer of the γ-glutamyl group from γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine, liberating 3-carboxy-4-nitroaniline. The catalytic concentration is determined from the rate of 3-carboxy-4-nitroaniline formation (Beleta and Gella, 1990). The analyses were carried out in the same assay session and the experimental groups were alternated in the reader plate. Repeatability of GGT

measurements, determined on a sub-sample measured twice, was high (R=0.99, N=19, P<0.001).

Carotenoids were quantified by diluting 60 µl of plasma in acetone (1:10 dilution). The mixture was vortexed and centrifuged at 11,000 ***g*** for 10 min to precipitate the flocculent proteins. The supernatant was examined in a ShimadzuUV-1603 spectrophotometer (Kyoto, Japan) and we determined the optical density at 446 nm. Finally, plasma carotenoid concentration (µg ml^{-1}) was calculated using a standard curve of lutein (Sigma-Aldrich, ref. 95507). Repeatability was determined on a sub-sample measured twice (R=0.99, N=20, P<0.001).

Carotenoid-based coloration

Digital photographs were analysed using Adobe Photoshop v 7.0. Red-legged partridges show considerable variability between birds in the relative proportion of bare skin around the eye covered by red pigmentation or showing the white skin underneath. The relative proportion of bare skin around the eye covered by red (carotenoid) pigmentation shows sexual dimorphism and is condition dependent in this species (Pérez-Rodríguez, 2008; Pérez-Rodríguez and Viñuela, 2008). Therefore, for each male, we calculated the percentage of pixels of the eye lore skin pigmented by carotenoids, thereby obtaining a measure of 'eye ring pigmentation' (%).

In addition, for each bird, we calculated the RGB components of the eye ring, nostril, upper mandible and lower mandible separately. The same components were calculated for the grey reference. Following previous studies on red-legged partridges (Pérez-Rodríguez et al., 2008; Mougeot et al., 2009b), the intensity of carotenoid-based red coloration (i.e. redness) was calculated as R divided by the average of R, G and B. 'Redness' values of the grey reference were used to correct for possible subtle differences in luminance between pictures.

For simplification and to adjust the analysis to the possible biological meaning of the coloured structures considered, we separated colour variables of the eye ring (eye ring pigmentation and eye ring redness) from those measured on the beak (nostril and upper and lower mandible redness), which are more keratinized structures. To evaluate overall beak redness, we conducted a principal component (PC) analysis on beak colour variables (nostril, upper mandible and lower mandible). The first PC explained 68% of variance, with nostril, upper mandible and lower mandible redness all having positive loadings (0.61, 0.60 and 0.51, respectively). We thus used PC1 scores as an index of overall beak redness. Measurements of eye ring pigmentation, eye ring redness and beak redness were repeatable (R=0.88, 0.83 and 0.84, respectively; all P<0.001; calculated on a subsample of 20 birds photographed twice).

Statistical analyses

We tested whether carotenoid-based coloration predicted the change in the level of TBARS or GGT (post- minus pre-treatment levels). For each response variable (change in TBARS or GGT), we performed a single general linear model (GLM), entering the following fixed effects: pre-treatment levels of the parameter analysed, treatment (control or PHA), eye ring pigmentation, eye ring redness, beak redness, and the interactions between treatment and colour variables. We explored whether variation in initial carotenoid levels or changes in circulating carotenoids during the experiment explained individual changes in lipid peroxidation or GGT levels by performing another two GLMs with change in TBARS or GGT as response variables. In both cases, pre-treatment levels of the parameter analysed, treatment, initial carotenoid levels and change in carotenoid levels (post- minus pre-treatment levels) were entered as fixed effects. PHA-injected birds, but not control birds, showed a decrease in circulating carotenoids during the experiment (Pérez-Rodríguez et al., 2008). Therefore, the interactions between treatment and initial carotenoid levels and between treatment and change in circulating carotenoids were also entered as terms in these two GLMs. In all models non-significant terms (P>0.05) were sequentially removed using a backward stepwise procedure and the statistics reported for them correspond to the step when they were removed from the model. All variables meet assumptions of parametric statistics. We failed to measure GGT in three samples and TBARS in another one, so sample sizes slightly differ between analyses.

RESULTS

The change in lipid peroxidation was significantly explained by eye ring pigmentation, but in a treatment-dependent manner (significant interaction; Table 1). The change in lipid peroxidation was negatively related to eye ring pigmentation in PHA-injected birds, but not in control birds (Fig. 1A). Although 'treatment' showed a significant effect in the final model (Table 1), this variable turned non-significant when its interaction with eye ring pigmentation was removed from the model ($F_{1,35}$=0.37, P=0.55). Neither beak nor eye ring redness was related to changes in oxidative damage (estimates ± s.e.: –0.02±0.11 and –0.07±0.11, respectively; Table 1).

The change in GGT activity was significantly and negatively related to eye ring pigmentation, the relationship being similar in

Table 1. Relationship between the expression of carotenoid-based traits and individual changes in lipid peroxidation and GGT in blood after cell-mediated immune challenge (PHA injection) or treatment with a control solution (PBS injection)

	Lipid peroxidation (TBARS) change			GGT change		
Factors	*F*	d.f.	*P*	*F*	d.f.	*P*
Initial levels	**88.7**	**1, 34**	**<0.001**	**9.08**	**1, 34**	**<0.01**
Treatment	**5.82**	**1, 34**	**0.02**	3.09	1, 33	0.09
Eye ring pigmentation	**5.04**	**1, 34**	**0.03**	**7.00**	**1, 34**	**0.01**
Eye ring redness	1.52	1, 33	0.22	0.06	1, 30	0.80
Beak redness	0.25	1, 31	0.62	0.00	1, 32	0.98
Eye ring pigmentation × treatment	**5.48**	**1, 34**	**0.02**	0.27	1, 28	0.61
Eye ring redness × treatment	0.31	1, 32	0.58	1.37	1, 29	0.25
Beak redness × treatment	0.16	1, 30	0.68	2.77	1, 31	0.11

Significant terms in final models are marked in bold. In the case of non-significant terms, statistics shown correspond to the step when they were removed from the model.

GGT, γ-glutamyl transferase; PHA, phytohaemagglutinin; PBS, phosphate buffer solution; TBARS, thiobarbituric acid reactive substances.

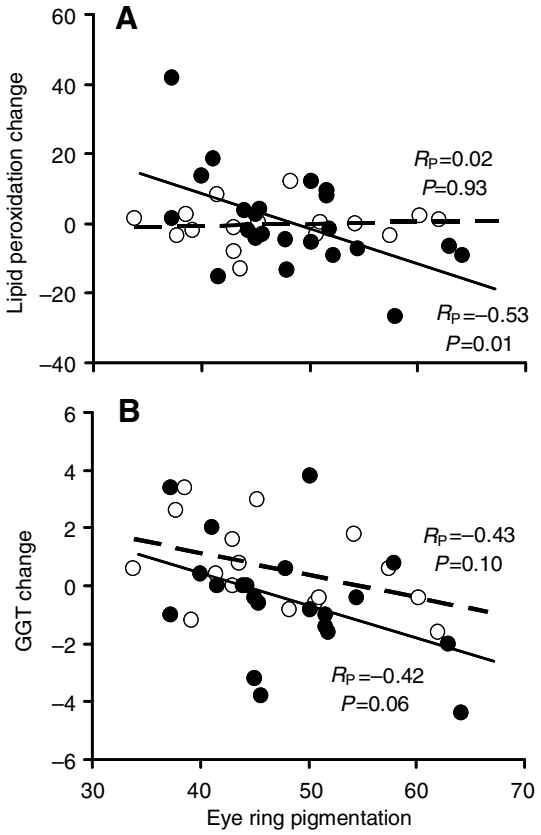

Fig. 1. Relationship between eye ring pigmentation and changes in the levels of (A) lipid peroxidation and (B) γ-glutamyl transferase (GGT) activity in blood. Values are raw residuals controlling for pre-treatment levels of each parameter. Control birds: open circles and dashed line; PHA-treated birds: filled circles and solid line.

the two treatments (Table 1, Fig. 1B). Eye ring and beak redness, however, did not show any relationship with changes in GGT (estimates ± s.e.: –0.18±0.15 and –0.04±0.15, respectively; Table 1). Changes in lipid peroxidation in pellet and changes in GGT activities in plasma, after controlling for initial levels, were not correlated to each other in any experimental group (PHA birds: R_P=0.05, P=0.82; control birds: R_P=–0.07, P=0.76).

Although circulating carotenoids decreased in PHA-injected birds (Pérez-Rodríguez et al., 2008), the change in TBARS was not explained by the change in circulating carotenoid levels ($F_{1,35}$=0.16, P=0.70; estimate ± s.e.: –0.05±0.12) or initial carotenoid levels ($F_{1,33}$=2.13, P=0.15; estimate ± s.e.: –0.17±0.12). These results did not differ between groups (i.e. non-significant 'treatment × change in carotenoids' and 'treatment × initial carotenoids' interactions: $F_{1,34}$=1.53, P=0.22 and $F_{1,32}$=1.16, P=0.29, respectively). Similarly, changes in GGT levels were not explained by changes in circulating carotenoid levels ($F_{1,32}$=0.09, P=0.77; estimate ± s.e.: –0.07±0.23) or initial carotenoid levels ($F_{1,33}$=1.74, P=0.20; estimate ± s.e.: –0.20±0.15). Again, these results did not differ between experimental groups (i.e. non-significant 'treatment × change in carotenoids' and 'treatment × initial carotenoids' interactions: $F_{1,30}$=0.22, P=0.64 and $F_{1,31}$=0.09, P=0.76, respectively).

DISCUSSION

We found that eye ring pigmentation predicted changes in oxidative damage, consistent with our initial hypothesis that carotenoid-based traits indicate an individual's capacity to tackle the oxidative stress associated with mounting a cellular immune response. Partridges with more pigmented eye rings not only mounted stronger immune responses after PHA injection (see Pérez-Rodríguez et al., 2008; Mougeot et al., 2009b) but also experienced less oxidative damage (measured as a change in lipid peroxidation) than less pigmented individuals. PHA-challenged birds showed a marked variability in immune response-induced oxidative damage: paler individuals suffered an increase in lipid peroxidation, whereas birds showing more pigmented eye rings not only avoided such damage but also tended to show a decrease in lipid peroxidation (Fig. 1A). In contrast, this relationship was not found in control birds, which did not face the immune challenge. Interestingly, the contrasting response of PHA-challenged birds depending on their degree of pigmentation cancelled changes in lipid peroxidation at the group level, resulting in an absence of overall differences between control birds and PHA-injected birds, as previously reported (Pérez-Rodríguez et al., 2008). However, previous studies have reported an increase in oxidative stress associated with a PHA immune challenge (Costantini and Dell'Omo, 2006; Hõrak et al., 2007), supporting the existence of an oxidative cost associated with the cell-mediated immune response (Costantini and Møller, 2009; Sorci and Faivre, 2009). Parasite challenges and infections were also shown to increase oxidative damage in another gallinacean, the red grouse *Lagopus lagopus scoticus* (Mougeot et al., 2009a; Mougeot et al., 2010), consistent with the idea that raising an immune response increases oxidative stress.

Consistent with our results, a recent study on red grouse found that the size of a similar carotenoid-based ornament before an experimental parasite infection predicted the magnitude of the subsequent change in parasite-induced oxidative damage (Mougeot et al., 2009a). Again, in red-legged partridges, eye ring coloration also revealed the individual's capacity to endure oxidative challenges in the past, as partridges maintaining redder eye rings at the end of reproduction were also those with less oxidative damage (TBARS) at that time (Alonso-Alvarez et al., 2008).

Eye ring pigmentation was also negatively related to changes in GGT activity, irrespective of the experimental treatment. GGT is an enzyme that, among other functions, is involved in the recycling of a key endogenous antioxidant (i.e. glutathione) in plasma in order to fight off intracellular oxidative stress (Lee et al., 2004). High GGT levels would therefore indicate an up-regulation of this recycling activity in response to a situation of oxidative stress (Shi et al., 1993; Lee et al., 2004; Lim et al., 2004; Yamada et al., 2006; Simao et al., 2008). Hence, the overall negative relationship that we found between eye ring pigmentation and changes in GGT levels suggests that more pigmented males were suffering less oxidative stress than less pigmented ones. However, besides this overall relationship between GGT and eye ring pigmentation, the condition of oxidative stress induced by our experimental treatment did not affect GGT levels, suggesting that this enzyme was not directly involved in individual resistance to oxidative challenge induced by PHA. The differential effects of the treatment on lipid peroxidation *versus* GGT (Fig. 1) are not surprising considering the complexities of the antioxidant–oxidative balance (e.g. Cohen and McGraw, 2009). The lack of correlation between changes in GGT and lipid peroxidation in any group could perhaps be due to the different biochemical pathways involved, as recycled glutathione should mostly be engaged in combating free radicals in the mitochondria, whereas lipid peroxidation occurs in any cell membrane (Halliwell and Gutteridge, 2007). Thus, lipid peroxidation could be influenced by other antioxidant molecules, masking the relationship between

the two parameters. Furthermore, the antioxidant responses include a wide array of up- and down-regulations, synergies and complex interactions between endogenous and exogenous compounds (e.g. Costantini and Verhulst, 2009; Pérez-Rodríguez, 2009), which may prevent us from finding intuitive and simple relationships. In any case, our results must be interpreted with caution because the role of GGT in oxidative stress, although empirically supported and recognized by recent studies in humans and mammals (Shi et al., 1993; Lee et al., 2004; Lim et al., 2004; Yamada et al., 2006; Simao et al., 2008), still awaits a proper assessment in birds.

Unlike eye ring pigmentation, eye ring redness did not predict changes in oxidative stress. Eye ring pigmentation and redness are positively but weakly related (Pérez-Rodríguez et al., 2008). It is therefore possible that eye ring pigmentation retained all the variance explained by overall eye ring ornamentation (which is determined by the amount of pigmented skin plus the redness of the pigmented area) in the models. Beak redness did not predict changes in oxidative stress either. Beak and eye ring coloration of red legged-partridges are dynamic traits showing seasonal changes in coloration and reaching their maximum redness during the breeding period (Pérez-Rodríguez, 2008). Also, both traits are related to body condition in this species (Pérez-Rodríguez and Viñuela, 2008; Mougeot et al., 2009b). Although neither beak nor eye ring coloration changed as a result of PHA injection during this experiment (Pérez-Rodríguez et al., 2008), eye ring pigmentation can rapidly mirror other physiological changes, such as sudden variations in the nutritional status of the individual (Pérez-Rodríguez and Viñuela, 2008). In contrast, beak redness seems to be a much more stable trait, and its coloration requires more time to change in response to changes in individual condition (Pérez-Rodríguez and Viñuela, 2008). This is consistent with the hypothesis that some carotenoid-based traits may be more labile than others, reflecting short-, medium- or long-term quality depending on the physiology of the tissue and its specific carotenoid turn-over (Pérez-Rodríguez and Viñuela, 2008; Mougeot et al., 2009b; Biard et al., 2009). It is therefore possible that individual resistance to an oxidative challenge, which relies on many factors that may vary on a short-term basis (e.g. intake of dietary antioxidants, recent oxidative challenges, etc.), are better reflected by a skin trait like the eye ring than by a more keratinized (and probably less labile) structure like the beak.

The underlying mechanism for the relationship between carotenoid-based ornaments and resistance to oxidative stress is still debated (reviewed in Pérez-Rodríguez, 2009). Carotenoids might act as significant antioxidants *in vivo*, thus directly linking ornament pigmentation to resistance to oxidative stress, since more pigmented individuals usually circulate more carotenoids (e.g. Blount et al., 2003; Martínez-Padilla et al., 2007; Pérez-Rodríguez et al., 2008). Male partridges injected with PHA showed a 13% decrease in circulating carotenoids within 24 h (Pérez-Rodríguez et al., 2008). However, neither initial carotenoid levels nor the change in levels during the experiment explained the changes in lipid peroxidation that occurred during the immune challenge. Similarly, changes in GGT levels, although related to eye ring pigmentation, were not explained by initial levels of carotenoids or by their change. Therefore, although birds with more pigmented eye rings circulate more carotenoids (Pérez-Rodríguez, 2008; Pérez-Rodríguez et al., 2008; Mougeot et al., 2009b), the antioxidant properties of carotenoids *per se* do not seem to mediate their higher resistance to oxidative damage. This would indicate that other antioxidants (endogenously produced or derived from diet but not analysed here) were mediating the higher resistance to oxidative damage of partridges with more pigmented eye rings. In fact, the antioxidant properties of carotenoids have been questioned recently, and alternative links between carotenoid-based ornaments and oxidative stress are considered (Pérez-Rodríguez, 2009). For instance, carotenoids might not act as efficient free radical scavengers *in vivo*, but could be sensitive to the effect of free radicals that bleach them. In this case, carotenoid-based coloration would be indicative of the ability of the antioxidant system to protect relevant bio-molecules (together with carotenoids) from oxidative damage (Hartley and Kennedy, 2004). However, carotenoids themselves would not be responsible for the antioxidant efficiency highlighted by the pigmented trait.

In conclusion, our results support the hypothesis that carotenoid-based coloration predicts an individual's capacity to tackle the oxidative stress associated with raising an immune response, and that carotenoid-based ornaments honestly signal an individual's capacity to protect itself effectively from oxidative stress (Hartley and Kennedy, 2004). Given that oxidative stress is a significant constraint in many biological processes (Costantini, 2008; Monaghan et al., 2009), carotenoid-based traits may honestly signal how individuals deal with these constraints.

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