Cell-mediated immune activation rapidly decreases plasma carotenoids but does not affect oxidative stress in red-legged partridges (*Alectoris rufa*)

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SUMMARY

In animals yellow-orange-red sexual traits pigmented by carotenoids have been suggested to act as signals of current health. Because carotenoids have important physiological functions, individuals might trade-off allocating these pigments to self-maintenance *versus* coloration. Carotenoids may act as scavengers of free radicals that are released during an immune response. Here, we experimentally assessed whether a local cell-mediated immune response affects circulating carotenoids, antioxidant status, oxidative damage and the expression of a carotenoid-based trait. Male red-legged partridges (*Alectoris rufa*) were subcutaneously injected with phytohaemagglutinin (PHA) or with phosphate buffer solution (controls). The effect of the treatment on circulating carotenoids, total plasma antioxidant status (TAS), lipid oxidative damage in erythrocytes (TBARS) and ornamentation was assessed. Immune challenge induced a 13% decrease in circulating carotenoids within 24 h. However, this treatment did not affect TAS, TBARS or coloration. Coloration, circulating carotenoids and cell-mediated immune response were positively correlated, but these were not related to TAS or TBARS. Carotenoids were only weakly related to TAS after controlling for the effect of uric acid levels. These results suggest that carotenoid-based ornaments may honestly indicate immunocompetence but probably not antioxidant capacity in this species, and that carotenoids might be relatively weak antioxidants in the plasma. Furthermore, even a relatively harmless and locally elicited immune challenge had important effects on circulating carotenoids, but this effect did not appear to be associated with oxidative stress. Alternative mechanisms linking carotenoids to immunity (not necessarily relying on the use of these pigments as antioxidants) should be considered in future studies on birds.

Key words: antioxidants, immune response, phytohaemagglutinin, red-legged partridge, sexual selection, TAS, TBARS.

INTRODUCTION

Carotenoid pigments are responsible for many bright yellow, orange and red colours in animals that often signal individual quality and therefore may have evolved through sexual selection (reviewed in Møller et al., 2000; Hill and McGraw, 2006). To disentangle how and why carotenoid-based ornaments may have evolved and are maintained as honest sexual or social signals, the mechanisms and costs associated with ornament expression must be understood (Andersson, 1994).

Apart of their potential value as signals of foraging capacity (they cannot be synthesized *de novo* by animals but must be acquired through the diet), it has been suggested that carotenoids may have antioxidant and immune-enhancing properties that may confer another way of honesty to carotenoid-based signals (Lozano, 1994; Olson and Owens, 1998; Møller et al., 2000; Blas et al., 2006). Because of their molecular composition, carotenoids may act as effective scavengers of free radicals, such as reactive oxygen and nitrogen species (Krinsky, 1989). These free radicals are released during immune responses and help to counter invading pathogens (Halliwell and Gutteridge, 1999). However, the toxicity of free radicals is not restricted to the pathogen and their overproduction can damage host tissues, leading to a situation of oxidative stress. Carotenoids used for antioxidant protection would no longer be

available for signal expression, and therefore carotenoid-based ornaments have been hypothesized to act as honest individual signals of health, oxidative stress or antioxidant defences (Lozano, 1994; Olson and Owens, 1998; Møller et al., 2000; Hartley and Kennedy, 2004). However, there is scarce evidence supporting that carotenoids have important antioxidant function *in vivo* (see Costantini and Møller, 2008), at least as compared with other antioxidants such as vitamins A, C and E or uric acid (Hartley and Kennedy, 2004). In fact, studies assessing whether immune system activation leads to oxidative damage in birds have yielded contradictory results (Alonso-Alvarez et al., 2004; Bertrand et al., 2006; Costantini and Dell'Omo, 2006; Hõrak et al., 2006; Hõrak et al., 2007).

Recent studies have shown that a humoral immune challenge (intraperitoneal injection of sheep red blood cells, SRBC) decreased circulating carotenoids (McGraw and Ardia, 2003; Peters et al., 2004; Aguilera and Amat, 2007) (but see Hõrak et al., 2006) and carotenoid-based coloration (Faivre et al., 2003; McGraw and Ardia, 2003; Peters et al., 2004). Similarly, the intraperitoneal injection of lipopolysaccharide (LPS) of *Escherichia coli*, which promotes an inflammatory response followed by antibody production, decreases plasma carotenoids (Koutsos et al., 2003; Alonso-Alvarez et al., 2004) and carotenoid-

based coloration (Alonso-Alvarez et al., 2004). Taken together, these studies support the hypothesis that mounting an immune response may drain carotenoids from the blood stream. However, rarely are the potential effects of immune challenge on plasma carotenoids, antioxidant defences and oxidative stress-induced damage measured concurrently [see Hõrak et al. (Hõrak et al., 2007) for a discussion of the importance of this point], which makes it difficult to ascertain the sequence of immune response activation, oxidative stress, decrease in circulating carotenoids and subsequent decrease in carotenoid-based ornamentation. In addition, the above-mentioned SRBC or LPS challenge protocols promote a systemic response that may be activated for weeks. These are only specific types of a wide array of immune responses that vertebrates activate to prevent and combat an infection. In most cases, pathogens are fought at the site of entrance into the body *via* local inflammatory and cellular responses with scarce systemic implications (Roitt et al., 2001). However, the possible effect of such a local immune response on circulating carotenoids and coloration remains poorly known (Costantini and Dell'Omo, 2006; Hõrak et al., 2007).

The phytohaemagglutinin (PHA) skin test is probably the most popular and widespread measure of immunocompetence used by avian ecologists (Smits et al., 1999; Martin et al., 2006; Kennedy and Nager, 2006). A subcutaneous injection of PHA promotes an inflammatory response encompassing T-cell mediated infiltration of granulocytes, macrophages and lymphocytes, in a complex and dynamic process that involves both innate and acquired components of the immune system (Martin et al., 2006). As a result, a small but measurable swelling is produced during the following hours at the site of injection, the magnitude of which is used as an index of cell-mediated immunocompetence. Although PHA-induced skin swelling is not a cost-free reaction (e.g. Alonso-Alvarez and Tella, 2001), it seems to elicit a more local response (compared with LPS or SRBC intraperitoneal injection) with few relevant systemic effects (Merino et al., 1999; Hõrak et al., 2000).

In this study, we analyzed the effect of a local cell-mediated immune response (promoted by the intradermal injection of PHA) on plasma carotenoids, total antioxidant status (TAS), oxidative damage (TBARS, a measure of lipid peroxidation) and carotenoid-based coloration in male red-legged partridges (*Alectoris rufa* Linnaeus), a species with conspicuous carotenoid-based ornaments (red coloration of eye rings and bill). We assigned males to one of two treatments: subcutaneous injection of phosphate-buffered saline (PBS), as a control, or injection of PHA to elicit a cell-mediated immune response. We hypothesized that immune challenge would lead to oxidative damage (i.e. increase in TBARS) and possibly also a decline in TAS. Alternatively, the immune challenge could promote the mobilization of antioxidant defences and cause an increase in TAS. In addition, if carotenoids have an important antioxidant function, we predicted that plasma carotenoids would change in the same way as TAS does. Given a possible trade-off between carotenoid allocation to coloured signals or to antioxidant defences, we also predicted that carotenoid-based ornamentation would decrease in the PHA-challenged group as compared with the control group. We also explored correlatively the relationships between TAS, TBARS and carotenoids in order to assess the relative contribution of these pigments to antioxidant defences of the individual. Finally, we evaluated whether carotenoid-based ornaments in the red-legged partridge may act as honest signals of immunocompetence or antioxidant defences, by exploring the correlations between

carotenoid-based coloration, cell-mediated immunity, TAS and TBARS.

MATERIALS AND METHODS
General procedure

We conducted the experiment at the experimental facility of the *Instituto de Investigación en Recursos Cinegéticos* (Ciudad Real, central Spain), *Dehesa de Galiana,* in December 2006 on 40 6-month-old male red-legged partridges hatched and reared in captivity. At this age, males have reached their definitive adult size, are sexually mature and have developed their conspicuous carotenoid-based coloration. During the experiment, birds were housed individually in outdoor cages [for further details on rearing and housing conditions see Pérez-Rodríguez et al. (Pérez-Rodríguez et al., 2006)] and fed *ad libitum* with commercial food pellets containing 5.26 μg 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 captive reared partridges (Blas et al., 2006; Pérez-Rodríguez et al., 2007; Pérez-Rodríguez, 2008).

On 11 December, after 2 weeks of adaptation to cages, we took a blood sample (0.8 ml) from the brachial vein of the right wing of each male using a heparinized syringe. Blood was stored at 4°C and centrifuged at 7000 ***g*** within 4 h of collection. Plasma and pellets were then separated and stored at –80°C until analysis. We also took a high resolution (2272×1704 pixels) digital photograph of the left side of the head to measure bill and eye ring redness. All pictures were taken under fluorescent light and against a grey standard background. The distance from the camera (Nikon Coolpix 4500) to the bird was held constant (40 cm) and a grey standard reference (Kodak Gray Scale, Kodak, New York, USA) was placed in all pictures next to the head of the bird.

On 14 December, birds of the PHA group (*N*=23) were injected subcutaneously in the patagium of the left wing with 0.5 mg of PHA (Sigma-Aldrich, Steinheim, Bayern, Germany; ref L-8754) suspended in 0.1 ml of PBS (Smits et al., 1999). Control birds (*N*=17) were injected with the same volume of PBS. Before injection, the thickness of the patagium was measured with a digital spessimeter (Mitutoyo Absolute 547-315, Kawasaki, Kanagawa, Japan) to the nearest 0.01 mm. After 24 h, patagium thickness was again measured at the point of injection and the difference between initial and final measurements in PHA-injected birds was considered as the cell-mediated immune response (Smits et al., 1999). In both cases (before and 24 h after injection) three measures of patagium thickness were taken. Both initial and final measurements were repeatable (intraclass correlation coefficients: r=0.99, $F_{23,48}$=510.3, $P<0.001$, and r=0.99, $F_{23,48}$=336.2, $P<0.001$, respectively) and therefore we used average values of the three measurements in the analyses. At the time of post-injection measurement (15 December), we collected a second blood sample from the brachial vein of the right wing as described above. Finally, a second set of digital photographs was taken on 16 December, 48 h after PHA or PBS injection, in a random subsample of 29 birds (18 PHA and 11 control birds).

Although plasma carotenoids do not show diel variation (Pérez-Rodríguez et al., 2007), other blood metabolites and cell-mediated immune responses may (Martínez-Padilla, 2006; Pérez-Rodríguez et al., 2008). For this reason, all blood samples and immune measurements were taken at the same time (1200-1500 h).

Laboratory analysis

TAS is a measure of the capacity of the plasma to quench free radicals, and is primarily the result of the pooled effect of all

extracellular antioxidant compounds of the blood (i.e. uric acid, vitamin E, vitamin C, carotenoids). TAS of plasma was assessed by means of commercial kits (Randox Laboratories Ltd, Crumlin, Northern Ireland, UK; ref. NX2332) in an A25 BioSystems spectrophotometer autoanalyzer (BioSystems S.A., Barcelona, Spain). Plasma samples were incubated for 15 s with a chromogen composed of metmyoglobin and ABTS® [2,2-azino-di-(3-ethylbenzthiazoline sulphonate). Then, hydrogen peroxide (H_2O_2) was added and the samples incubated for 195 s. H_2O_2 addition induces the production of the radical cation ABTS®, which generates a blue-green colour. Colour change was determined by measuring at 600 nm before and after H_2O_2 addition.

TAS values are frequently related to the level of circulating uric acid (Cohen et al., 2007; Hõrak et al., 2007) which may confound interpretation of results. Uric acid is the main form of nitrogen excretion in birds and high levels of TAS could be an indication of incidental amino acid catabolism (i.e. high uric acid levels) rather than regulated antioxidant protection (Cohen et al., 2007). Therefore, we measured uric acid in all plasma samples in the same autoanalyzer and by means of commercial kits (Biosystems SA, Barcelona, Spain; ref. 11521), following the uricase/peroxidase procedure (Fossati et al., 1980).

Lipid peroxidation in red blood cells was assessed following the method of Aust (Aust, 1985). The principle is based on the fact that most tissues contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. Blood pellets were thawed 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 sample (1 m) of that 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 results with those obtained from a curve with different malondialdehyde concentrations [i.e. the end products of lipid peroxidation (Aust, 1985)]. TAS and TBARS measurements were significantly repeatable (r=0.94 and r=0.76, respectively; C.A.-A., unpublished).

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 the optical density at 446 nm [the wavelength of maximal absorbance for lutein (Mínguez-Mosquera, 1993)] was determined. Finally, plasma carotenoid concentration (μg ml^{-1}) was calculated using a lutein standard curve (Sigma-Aldrich, ref. 95507).

Carotenoid-based coloration
We analysed digital photographs using Adobe Photoshop v 7.0. For each male, we calculated the RGB (red, green, blue) components of the eye ring, nostril, upper mandible and lower mandible, separately. The same components were calculated for the grey reference. Following previous studies with this (Villafuerte and Negro, 1998) 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. 'Redness' values of the grey reference were used to standardize all colour measurements and correct for possible subtle differences in luminance between pictures. The average redness of the grey reference in the entire picture set was calculated. For each photographs, the difference between the redness of the standard and the average was subtracted from the redness of the ornaments in order to control for any subtle variation in illumination between photographs.

There exists a considerable variability between birds in the relative proportion of the bare white skin around the eye that has red (carotenoid) pigmentation. This is related to sexual dimorphism and is condition-dependent in this species (Pérez-Rodríguez, 2007). Therefore, for each male, we calculated the percentage of pixels of the eye lore skin pigmented by carotenoids (hereafter referred to as eye ring pigmentation) also using Adobe Photoshop v 7.0.

Statistical analyses
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 redness and eye ring pigmentation) from those measured on the bill (nostril and upper and lower mandible redness), which are more keratinized structures. This separation is meaningful since eye rings are soft tissues and therefore may change colour more rapidly than bill. To evaluate overall bill redness, we conducted a principal component analysis on bill colour variables (nostril, upper mandible and lower mandible). The first principal component (bill PC1) explained 68% of variance, with nostril, upper 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 bill redness. We explored relationships between carotenoids, ornamentation and cell-mediated immune response using Pearson correlations. The effect of PHA-induced response on carotenoids or colour variables was tested using general linear mixed models, with sampling time (before or 24 h after injection) and treatment (PHA or PBS) as a fixed factors and individual as a random variable. All variables were normally distributed (Shapiro–Wilk tests) and all tests were two-tailed.

RESULTS
Measures of oxidative stress before immune challenge
Uric acid concentration was strongly, positively associated with TAS (r_P=0.60, N=40, P<0.001). By contrast, TAS was not significantly related to plasma carotenoids (r_P=0.27, N=40, P=0.087). After removing the effect of uric acid (by entering it as a covariate), TAS was only marginally, positively, related to plasma carotenoids ($F_{1,37}$=3.75, P=0.06; R^2=0.42). Overall, plasma carotenoids explained only ~6% of the variance in TAS after controlling for uric acid concentration. TBARS of red blood cells were not significantly explained by TAS (alone or after controlling for uric acid concentration), uric acid or by plasma carotenoids (all P>0.75).

Plasma carotenoids, carotenoid-based coloration and cell-mediated immunity
Eye ring redness positively correlated with eye ring pigmentation (Table 1). All other correlations among colour variables were non-significant. Plasma carotenoid levels before experimental treatment were significantly and positively related to eye ring pigmentation and bill redness, but not with eye ring redness (Table 1). TAS and TBARS before experimental treatment were not related to any colour trait (all P>0.25).

In PHA-injected birds, cellular immune response (wing web swelling at 24 h) showed a positive association with plasma carotenoids before immune challenge (Table 1, Fig. 1). Furthermore,

Table 1. *Correlations between plasma carotenoids, carotenoid-based ornamentation before experimental treatment (see Methods) and cell-mediated immune response*

	Eye ring pigmentation			Eye ring redness			Bill redness (PC1)			CMI		
	r_P	N	P	r_P	N	P	r_P	N	P	r_P	N	P
Carotenoids ($\mu g\ ml^{-1}$)	0.50	40	0.001	0.15	40	0.34	0.3	40	0.05	0.42	23	<0.05
Eye ring pigmentation				0.48	40	<0.01	0.19	40	0.21	0.49	23	<0.05
Eye ring redness							0.22	40	0.16	0.64	23	<0.001
Bill redness (PC1)										0.18	23	0.39

PC1, first principal component; CMI, cell-mediated immune response, measured as wing web swelling, in mm.

both eye ring redness and pigmentation before the experiment, but not bill redness, were significantly related to PHA response (Table 1, Fig. 1). TAS and TBARS before immune challenge were not related to the strength of the response (both $P>0.93$).

Effect of PHA on carotenoid-based coloration, plasma carotenoids and oxidative stress

Before PHA or PBS injection, none of the analyzed variables differed between experimental and control birds (ANOVA, all

Fig. 1. Relationship between cell-mediated immune response (CMI; wing web swelling; in mm) and (A) plasma carotenoids ($\mu g\ ml^{-1}$), (B) eye ring pigmentation (%) and (C) eye ring redness.

$P>0.124$). Body mass did not change during the study (time: $F_{1,40}=0.95$, $P=0.33$) and was not affected by the experimental treatment (time $\times$ treatment interaction: $F_{1,40}=0.22$, $P=0.64$). 24 h after challenge, PHA-injected birds showed on average a 13% decrease in plasma carotenoids (range +3.0% to –33.5%), whereas PBS-injected birds showed no significant change (Fig. 2). These changes in plasma carotenoids over time differed significantly between groups (significant time $\times$ treatment interaction; Table 2). By contrast, no effect of immune challenge was detected for TAS, TBARS or uric acid (Table 2, Fig. 2). Results did not change when the effect of uric acid on TAS was controlled for (by entering it as a covariate), but the common tendency to increase during the experiment became non-significant (Table 2).

Despite the clear effect of PHA-induced immune response on circulating carotenoids, none of the colour variables was affected by experimental treatment (Table 2). However, eye ring and bill redness (PC1) increased during the study in control and PHA-challenged birds.

Among PHA-injected birds, neither absolute nor relative (percentage of initial plasma carotenoids) change in plasma carotenoids was related to the intensity of cell-mediated immune response ($r_P=-0.19$, $N=23$, $P=0.35$ and $r_P=-0.10$, $N=23$, $P=0.62$, respectively). Thus, we had no evidence that the greater cellular immune responses consumed more carotenoids than weaker ones.

DISCUSSION

Although initially considered as indicators of foraging efficiency and body condition, in recent years it has been suggested that carotenoids may be more related to an allocation trade off between immune-related functions and ornamentation, thus leading to an alternative way of honest carotenoid-based signalling (Lozano, 1994; von Schantz et al., 1999; Møller et al., 2000; Blas et al., 2006). The underlying hypothesis is that carotenoids have antioxidant properties and that the activation of an immune response leads to a situation of oxidative stress that may force a reallocation of available carotenoids for quenching free radicals rather than for ornament pigmentation. As a result, ornament expression is expected to decrease in challenged individuals (Lozano, 1994; von Schantz et al., 1999; Møller et al., 2000). Therefore, apart from foraging efficiency, carotenoid based ornaments might indicate immunocompetence, health and antioxidant status. This study gives mixed support for this hypothesis.

We found that male red-legged partridges with more intense carotenoid-based coloration (specifically, redder and more extensively pigmented eye rings) mounted stronger cellular responses after intradermal injection of PHA. However, carotenoid-based coloration, although positively associated with higher levels of circulating carotenoids, was not related to TAS or TBARS. Furthermore, cell-mediated immune response correlated with circulating carotenoids, but not with TAS or TBARS. These results
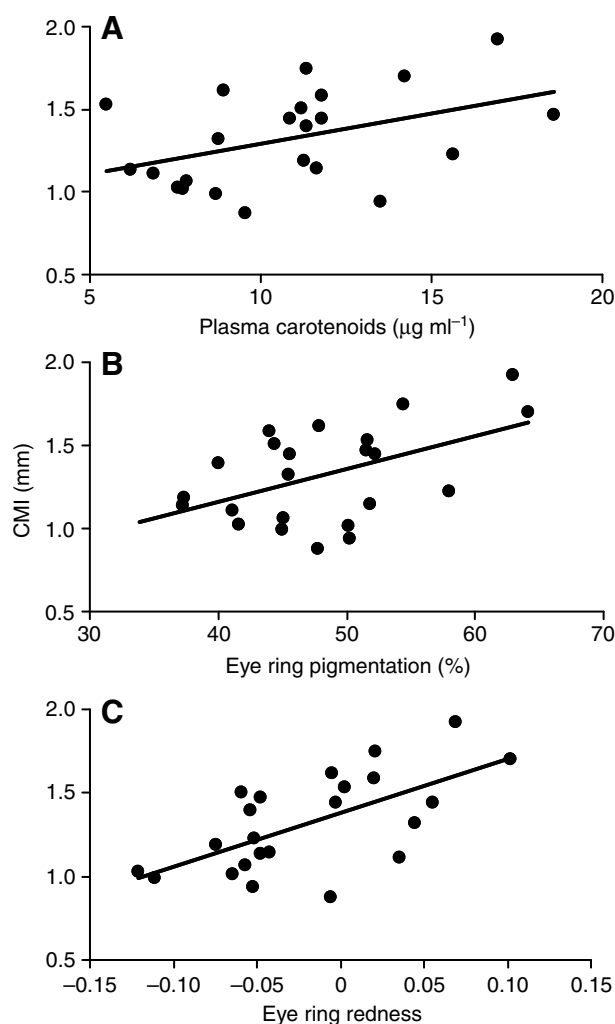
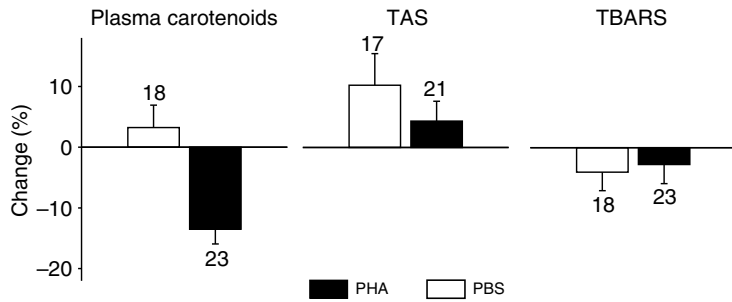Plasma carotenoids   TAS   TBARS

Fig. 2. Change (% of pre-injection values) in circulating carotenoids, plasma TAS (total antioxidant status) and red-blood cell TBARS (thiobarbituric acid reactive substances; a measure of oxidative damage) 24 h after PHA (phytohaemagglutinin; filled columns) or PBS (control; open columns) injection. Values are means ± s.e.m. Numbers above or below bars indicate sample size.

are consistent with the hypothesis that carotenoid-based ornaments may honestly signal immunocompetence (Blount et al., 2003; McGraw and Ardia, 2003; Peters et al., 2004; Mougeot, 2008), but not antioxidant defences, as recently suggested (Hartley and Kennedy, 2004). In addition, we found that the contribution of plasma carotenoids to TAS was small and they were not related to TBARS. An endogenous metabolite, uric acid, explained 36% of the variance in TAS, similar to the findings of recent studies (Cohen et al., 2007; Hõrak et al., 2007). Only when uric acid was statistically controlled for, were plasma carotenoids weakly (marginally) related to TAS, explaining a further 6% of variation. Thus, the hypothetical role of carotenoids as powerful antioxidants *in vivo* is questionable and should not be assumed "*a priori*" (Costantini et al., 2006; Costantini et al., 2007; Isaksson et al., 2007; Costantini and Møller, 2008).

We found that T-cell-mediated immune activation reduced circulating carotenoids. This decrease was marked (average 13% of initial circulating levels) and fast, as it was detected as soon as 24 h after PHA injection. A decrease in plasma carotenoids (and, eventually, carotenoid based ornamentation) as a result of mounting a systemic immune response (intraperitoneal injection of SRBC or LPS) has been reported by previous studies (Faivre et al., 2003; McGraw and Ardia, 2003; Peters et al., 2004; Alonso-Alvarez et al., 2004; Aguilera and Amat, 2007). However, the effect of a more local challenge, such as the intradermal injection of PHA in the wing patagium, is much less known. Recently, a study on zebra finches (*Taeniopygia guttata*), found a similar decrease in circulating carotenoids after PHA injection (McGraw and Ardia, 2007), although no control (PBS injected) birds were used in that study. By contrast, an increase in circulating carotenoids have been found in Eurasian kestrel (*Falco tinnunculus*) nestlings 24 h after PHA injection (Costantini and Dell'Omo, 2006), whereas no change in circulating carotenoids was detected 72 h after PHA challenge in

adult greenfinches (*Carduelis chloris*) (Hõrak et al., 2007). These inconsistencies between studies may be due to differences in timing of sampling (Hõrak et al., 2007) or to differences among taxa, or age groups in carotenoid allocation priorities or in the ability to buffer the impact of cell-mediated immune challenge. The growing body of literature on this subject will allow assessing the relative importance of these factors in the future.

Despite an important decrease in plasma carotenoids, we did not find any effect of cell-mediated immune activation on TAS or TBARS. Intradermal injection of PHA rapidly induces an inflammation and local infiltration of several cell types (thrombocytes, basophils, eosinophils, heterophils, lymphocytes, macrophages) (Martin et al., 2006). Once activated, heterophils and macrophages produce reactive nitrogen and oxygen species (Koner et al., 1997; Nathan and Shiloh, 2000; Coleman, 2001) that are expected to affect the antioxidant machinery of the individual. In fact, unlike our study, the above mentioned recent studies reported an increase in lipid peroxidation and free radical production accompanied by a decrease or increase, respectively, in total antioxidants after PHA injection (Costantini and Dell'Omo, 2006; Hõrak et al., 2007).

How can the marked reduction in available carotenoids, despite the absence of any effect on TAS or TBARS, be explained? One possible explanation is that the antioxidant properties of carotenoids buffered the free radical production associated with the activation of a local immune response, thus preventing any oxidative damage. However, as stated above, the relative contribution of plasma carotenoids to TAS was weak. Alternatively, if carotenoids were significant antioxidants, the 13% decrease in circulating levels after PHA injection should have negatively affected TAS. Instead of such a decrease, we found that TAS tended to increase during the study, in all birds (a pattern attributable to the weak generalized tendency to increase uric acid levels during the experiment). The

Table 2. Results of the general linear mixed models for plasma carotenoids, uric acid, TAS, TBARS and colour variables in relation to experimental treatment

	Time			Treatment			Time×treatment		
	F	d.f.	P	F	d.f.	P	F	d.f.	P
Carotenoids	10.0	1,40	<0.01	0.29	1,40	0.59	18.2	1,40	<0.001
Uric acid	3.38	1,31.5	0.07	2.09	1,32.7	0.15	0.02	1,31.5	0.89
TAS	5.50	1,39.2	<0.05	0.40	1,39.2	0.53	1.42	1,39.2	0.24
TAS (UA controlled)*	2.88	1,36.1	0.09	0.08	1,34.8	0.78	2.52	1,35	0.12
TBARS	3.01	1,79	0.08	0.24	1,79	0.62	0.14	1,79	0.71
Eye ring pigmentation	0.17	1,28.3	0.67	0.02	1,39	0.89	0.04	1,28.3	0.83
Eye ring redness	46.35	1,26	<0.001	0.60	1,37.9	0.44	0.13	1,26	0.72
Bill redness (PC1)	82.7	1,26.1	<0.001	0.02	1,35.3	0.87	0.00	1,26.1	0.94

TAS, total antioxidant status; TBARS, thiobarbituric acid reactive substances (a measure of oxidative damage); PC1, first principal component.
*Effect of uric acid (UA) statistically controlled by entering it as a covariate ($F_{1,72.4}$=40.6, P<0.001).
Sampling time (before or after) and treatment [PHA (phytohaemagglutinin) or PBS] are fixed factors. Individual was entered as a random variable.

absence of any effect of carotenoid depletion on TAS or TBARS may be explained by a compensatory increase in circulating levels of non-measured antioxidants (such as vitamin E, vitamin C, enzymes, etc) (Vider et al., 2001; Tauler et al., 2006; Hõrak et al., 2007).

Another possible explanation for the effect of cell-mediated immune challenge on plasma carotenoids irrespective of oxidative stress is that plasma carotenoids are poor antioxidants *in vivo* (Hartley and Kennedy, 2004; Costantini and Møller, 2008) but are particularly sensitive to the negative effect of free radicals (that is, free radicals have a deeper impact on plasma carotenoids than *vice versa*). Carotenoids can be attacked at many different sites at their long conjugated aliphatic chains, so it is likely that even low concentrations of free radicals (with no significant effect on TAS or TBARS) may have an important impact on circulating carotenoids, which lose their pigmentary properties after being attacked by oxidants. If so, carotenoids would not be signalling antioxidant ability, as our result suggest, but could be short-term indicators of changes in oxidative stress.

A third possibility is that plasma carotenoids are required for mounting an immune response, but perform functions other than antioxidant protection. In fact, carotenoid supplementation usually enhances immune response (Blount et al., 2003; McGraw and Ardia, 2003; Aguilera and Amat, 2007) and, conversely, immune response depletes available carotenoids (McGraw and Ardia, 2003; Alonso-Alvarez et al., 2004; Aguilera and Amat, 2007) (this study). By contrast, carotenoid supplementation does not always increases antioxidant protection (for a review, see Costantini and Møller, 2008), and the effect of immune activation on oxidative stress has yielded contradictory results (Alonso-Alvarez et al., 2004; Bertrand et al., 2006; Costantini and Dell'Omo, 2006; Hõrak et al., 2006; Hõrak et al., 2007). It seems therefore that, with the information currently available, the link between carotenoids and immunity is sounder than that between these two factors and oxidative stress. Therefore, alternative links between immunity and carotenoids, not necessarily based on the antioxidant properties of these pigments, should be explored and considered in future studies. For instance, induction of acute phase response in chickens depletes carotenoids in plasma but increases their concentration in thymocytes (Koutsos et al., 2003), whose activation requires carotenoid derivatives as cofactors (Garbe et al., 1992). Furthermore, carotenoids and some of their derivatives are involved in the expression of immune-related genes (Geissmann et al., 2003), up regulation of proteins involved in cell-to-cell communication (Basu et al., 2001) or increase membrane fluidity (Chew and Park, 2004), functions of vital importance when mounting an immune response.

The clear reduction in plasma carotenoids induced by PHA injection did not translate into rapid changes in carotenoid-based coloration. In fact, we found that eye ring and bill redness significantly increased during the 5-day period between measurements, to a similar extent in both control and PHA-treated birds. This increase is consistent with the seasonal variation of carotenoid-based coloration described for this species (Pérez-Rodríguez, 2008), so a possible explanation for the lack of effect of the treatment is that individuals were experiencing an allocation trade off in the use of available carotenoids (ornamentation *vs* immunity) and prioritized investment in sexual signalling because of the proximity to the reproductive season. Another possible explanation is that 48 h is too short a period to observe any such effect on coloration, or that the decrease in circulating carotenoids was too small to exert an impact. Although such a rapid response

in carotenoid based coloration has been observed in other species (Velando et al., 2006; Rosen and Tarvin, 2006), the ornaments of the red-legged partridge might require more time to reflect changes in circulating carotenoids. In addition, as the red coloration of our study species is mainly based on asthaxanthin (R. Mateo, C.A.-A., L.P.-R. and J.V., unpublished data), ingested carotenoids cannot be used directly but have to be metabolized, thus implying a greater delay in colour responses. It is also possible that the local response elicited by PHA injection did not last long enough to have impact on coloration (the PHA stimulated swelling usually disappears after 2 days) (Smits et al., 1999). It should also be noted that subcutaneous injection of PHA only mimics a local attack of a potential pathogen. In case of a real infection, the cell-mediated immune response would be extended over time (and surely other arms of the immune system would be also activated) thus increasing the magnitude and duration of the drop in circulating carotenoids and its potential impact on coloration. For instance, in red grouse *Lagopus lagopus scoticus*, experimental reductions of nematode parasites were shown to increase cell-mediated immunity (Mougeot and Redpath, 2004) as well as circulating carotenoids and ornamental coloration (Martinez-Padilla et al., 2007; Mougeot et al., 2007), indicating that recurrent infections negatively affect cellular immunity and carotenoid availability and use.

In conclusion, our results suggest that carotenoid-based ornamentation may honestly indicate immunocompetence. By contrast, it does not appear to signal antioxidant capacity. In fact, the relative contribution of circulating carotenoids to plasma antioxidant defence was weak. Mounting a cell-mediated immune response depleted circulating carotenoids, but no effect on oxidative damage or antioxidant defences was detected. This suggests that the link between carotenoid ornamentation and immunity may not necessarily rely on the antioxidant properties of carotenoids or the oxidative stress associated with mounting a cellular immune response. Alternative mechanisms to explain the immunoenhancing properties of carotenoids are needed and should be considered in future studies. Given the complexity of the interactions between individual antioxidants, total antioxidant status, oxidative damage and immunity, further research is required to fully understand the relative roles of carotenoids and antioxidants in honest sexual signalling.

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