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Dynamics of PHA-induced immune response and plasma carotenoids in birds: should we have a closer look?

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SUMMARY

Allocation trade-offs of limited resources are thought to ensure the honesty of sexual signals and are often studied using controlled immune challenges. One such trade-off between immunity and ornaments is that involving carotenoids. Phytohemagglutinin (PHA)-induced immune response is a widely used immune challenge, yet more details on the underlying physiological mechanisms and potential costs are needed. We investigated the temporal dynamics of PHA-induced immune response and associated changes in blood carotenoids, body mass and a carotenoid-based coloured signal. We found variation in individual response patterns to PHA after peak swelling was reached, with birds showing either a rapid or a slow subsequent decrease in swelling, suggesting variation in the duration of the immune response and/or inflammation. Body mass did not affect immune response. Plasma carotenoids followed a transient decrease closely matching the dynamics of the swelling. The peak of the immune response was negatively related to initial plasma carotenoid levels and positively correlated to the relative decrease in plasma carotenoids. Individual variation in duration of the swelling could be partly explained by plasma carotenoids; high initial carotenoid levels were associated with a slower decrease of the swelling. These contradictory effects of carotenoids suggest a complex role in the immune response. Bill colour was positively correlated to initial plasma carotenoid concentration but it did not predict or change as a consequence of immune response to PHA. Bill colour thus reflects medium- or long-term quality rather than immediate quality. Taking into account the dynamics of the immune response and that of associated physiological parameters would thus yield new insights into our interpretation of variation in PHA response.

Key words: bill colour, blackbird, carotenoid-based signals, cell-mediated immune response, immunocompetence.

INTRODUCTION

The study of immunocompetence, i.e. the ability of an individual to prevent, control and clear infections and associated trade-offs has become, in recent years, a predominant field in evolutionary ecology. Immune responses entail evolutionary and life history trade-offs as well as proximate energetic costs and resource-based trade-offs (Sheldon and Verhulst, 1996; Schmid-Hempel, 2003). One such trade-off based on the allocation of limited resources to immunity or other competing functions is that involving carotenoids. Carotenoids are important immune-modulators and part of the antioxidant system (reviewed by Bendich, 1993; Edge et al., 1997; Møller et al., 2000; Surai, 2002). These pigments are also potentially limiting, so that carotenoids allocated to immune function might be drawn out of the pool available for developing carotenoid-based signals honestly revealing the quality of their bearer (Lozano, 1994; Shykoff and Widmer, 1996) (reviewed by Møller et al., 2000). Indeed, immune activation with various antigens eliciting inflammatory and/or humoral immune response has generally been reported to have negative effects on circulating carotenoids, quickly reducing their levels (Koutsos et al., 2003; McGraw and Ardia, 2003; Alonso-Alvarez et al., 2004; Peters et al., 2004) [but see Costantini and Dell'Omo and Hõrak et al. (Costantini and Dell'Omo, 2006; Hõrak et al., 2006)]. In addition, carotenoid-based bill colour relying on constant pigment deposition was shown to be negatively affected by immune responses induced by controlled immune challenge (Faivre et al., 2003a; Alonso-Alvarez et al., 2004; Peters et al., 2004; Gautier et al., 2008).

One widely used immune challenge in studies of immunocompetence in birds is the phytohemagglutinin (PHA)-induced skin swelling test, in which this plant lectin is injected subcutaneously. PHA first induces an acute response 4 h after injection, primarily characterised by oedema. Then a delayed-type hypersensitivity response is induced through stimulating heterophil, basophil, eosinophil, macrophage and thrombocyte cell infiltration in the dermis and dense perivascular infiltration of T-lymphocytes at the site of injection (Sharma, 1990; Parmentier et al., 1998; Martin et al., 2006). This late response generally peaks 18 h after injection and may last up to 36 h. This test therefore reflects the combined responses of T-cells, cytokines and inflammatory cells (Davison et al., 1996) and involves both innate and adaptive components of the immune system (Martin et al., 2006; Tella et al., 2008). An index of the immune reaction is given by the resulting skin swelling at the site of injection, which is generally measured after 24 h (Martin et al., 2006). PHA-induced immune response has been shown to trade-off with other functions and to correlate with individual condition and quality (reviewed by Martin et al., 2006). However, the use of the PHA test and its interpretation in terms of immunocompetence is currently being questioned, at least until we have gained a better knowledge of its relationship with parasite resistance and also of the induced immune response and physiological consequences (Kennedy and Nager, 2006; Owen and Clayton, 2007). Accordingly, recent studies in immuno-ecology have not only measured PHA response but at the same time several physiological parameters that might reveal its functional significance

and potential costs, such as oxidative stress and associated damages (Costantini and Dell'Omo, 2006; Hõrak et al., 2007; Pérez-Rodríguez et al., 2008).

However, another aspect of the immune response to PHA that has been relatively neglected until now, despite its biological relevance, is its temporal dynamics. Individuals may not only vary in the maximum response attained (peak response) but also in the latency to reach it and/or in how long they maintain the immune response. This may have consequences for the individual in terms of parasite resistance and fitness. All three components of the immune response might trade-off against each other, be subjected to different constraints and induce different benefits and costs for the individual. Another source of variation among individuals in immune response is senescence (Haussmann et al., 2005; Palacios et al., 2007). For example, in house sparrows *Passer domesticus*, sex and age influenced the number of immune cells recruited at the site of PHA injection (Martin et al., 2006), and stronger immune responses took longer to develop than weaker ones (Navarro et al., 2003). Experimentally increased levels of testosterone compromised the ability to maintain PHA response and humoral immune response to sheep red blood cells compared with controls in house finches *Carpodacus mexicanus* (Deviche and Cortez, 2005).

In line with the recent calls for a more detailed description of the PHA-induced immune response in order to refine its interpretation (Kennedy and Nager, 2006; Owen and Clayton, 2007), we investigated the temporal dynamics of PHA-induced immune response and a potential associated change in blood carotenoids, body mass and a carotenoid-based coloured signal. The underlying question being whether there was individual variation in response patterns and whether this variation might be related to physiological or morphological parameters. We chose the blackbird *Turdus merula* Linnaeus 1758 as a model species, as males in this sexually dimorphic species exhibit yellow to orange carotenoid-based bill colour, which has been previously shown to positively correlate with the intensity of PHA response and to reveal the current state of activation of the immune system (Faivre et al., 2003a; Faivre et al., 2003b). We first wanted to follow in detail the dynamics of the immune response to determine whether there would be more information to be gained from using indices of immune response later than 24 h after injection. Another aim of this study was to investigate the relationship between plasma carotenoids and immune response to PHA. In particular we wanted to determine whether basal carotenoid levels measured before immune challenge influenced the immune response. We might indeed expect a positive effect as increased carotenoid levels after dietary supplementation have been shown to result in higher immune response to PHA (Blount et al., 2003; McGraw and Ardia, 2003). In addition, if carotenoid levels were found to change following the immune challenge, we wanted to test whether relative changes in carotenoids were associated with the dynamics of the immune response. Lastly, we monitored changes in bill colour to assess its potential as a dynamic signal of quality as reflected by immune status. We expected bill colour to be correlated to plasma carotenoids and to predict the immune response. If bill colour were a rapidly variable trait we would also expect a parallel change in colour with any change in plasma carotenoids. Bill colour has been shown to decrease as quickly as within one week of immune challenge, probably following a decrease in plasma carotenoids (Faivre et al., 2003a; Peters et al., 2004). However, an increase in bill colour following an increase in plasma carotenoids has been shown after long-term (four weeks) dietary supplementation experiments, although detailed timing of these colour changes were not reported (Blount et al., 2003; McGraw and Ardia, 2003; Alonso-Alvarez et al., 2004).

MATERIALS AND METHODS

A total of 51 male adult blackbirds (two years old and older) were caught in mist-nets in botanical gardens of Dijon, France (47 deg.19′N, 5 deg.02′E) during winter 2006. Birds were brought back to the field station and housed in outdoor aviaries sized 220×150×250 cm (L×W×H) with six males in each. The birds were fed *ad libitum* with commercial food (specific granules for large turdid species, COFNA, Tours, France) and tap water. During the study, birds were held on the natural daylength cycle. The study was carried out in July 2006. Birds were released after the experiment. Males were randomly distributed between two experimental groups: challenged with PHA (N=33) or control-injected with phosphate-buffered saline (PBS, N=18). Birds were allocated randomly with regards to morphological characters, date of initial capture in the wild and previous aviary. Sample sizes differ between the two groups because the aim of the experiment was to investigate variation in immune response among challenged birds. In order to be able to detect variation and to reliably use parametric multiple regressions, the sample size in this group had to be at least 30 birds. In each aviary, we mixed challenged and control males.

Immune-challenged blackbirds were injected with 100 µl of 10 mg ml^{-1} PHA (PHA-P, Sigma-Aldrich, Lyon, France) dissolved in PBS in the centre of right wing web (patagium) (Smits et al., 1999; Faivre et al., 2003b). Blackbirds of the control group were injected with 100 µl of PBS. All individuals were injected on 17 July in the morning between 08:00 h and 12:00 h.

We measured the thickness of the patagium at the injection site just before injection (thereafter day 0) and 1, 2, 4, 7, 9 and 11 days after injection, using a pressure-sensitive spessimeter with an accuracy of 0.01 mm (Teclock SM-112, Alpa SpA, Milano, Italy). All captures and bird manipulations were performed at the same time of the day (i.e. between 08:00 h and 12:00 h) and in the same order between aviaries. This ensured that the same time elapsed between subsequent captures for all birds and that measurements for each bird were spaced by 24 h (or a multiple of 24 h) ±1 h. In order to limit the number of captures and associated stress, we planned not to measure skin thickness after 12 h and to measure thickness every two days after the measurements at 24 h and 48 h. However, on day 4 birds showed signs of stress (reduced activity) and measurements planned on day 6 were then delayed until day 7 (as birds then behaved normally). An assistant restrained the bird while the micrometer was placed over the injection site. Each wing was measured in duplicate. The repeatability of measurements was high and significant (intra-class correlation coefficient r=0.99, P<0.0001) (Lessells and Boag, 1987), and we subsequently used mean swelling in the analyses. Body mass was recorded using an electronic balance (Scout Pro SPU 202, Ohaus Corp., Pine Brook, NJ, USA) to the nearest 0.1 g on all occasions. After measuring the wing web thickness, a blood sample was collected from the brachial vein in a heparinised capillary. Blood was immediately centrifuged (1073 ***g***, 4°C, 15 min) and the plasma was stored in 1.5 ml Eppendorf tubes at –80°C for later analysis.

Bill colour was scored on the first and last capture. Colour was assessed by visual comparison with a Roche Yolk Colour Fan (Roche, Neuilly-sur-Seine, France). The colour scores, ranging from index 1 (pale yellow) to index 15 (orange), are characterised by tristimulus values of the CIE–1931-standard colorimetric system and have been shown to be consistently correlated to colour parameters obtained with a spectroradiometer (Faivre et al., 2003b). Scores were

always given by the same observer (J.M.) under the same light conditions blindly with respect to treatment group. Scorings were highly repeatable (see Baeta et al., 2008). Observed bill colour scores ranged from index 8 to index 11 with 0.5 intervals.

Blackbirds were immediately placed back in their aviary after measurements and blood sampling, i.e. within 5 min.

Determination of plasma carotenoid concentration

Plasma samples (30 µl) were first mixed with 60 µl ethanol, followed by antioxidants being extracted twice with 500 µl hexane. Hexane extracts were pooled and evaporated at ambient temperature under nitrogen flow and the residue was dissolved in 150 µl ethanol and kept on ice. The optical density of 100 µl of the re-suspended samples was read in 96-wells Microtitration plates (Sterilin, London, UK) at 450 nm with a spectrophotometer (Versamax, Molecular Devices, Sunnyvale, CA, USA). A standard solution of lutein, starting at 20 ng µl^{-1} and subsequently serially diluted in ethanol six times to 0.31 ng µl^{-1}, with pure ethanol as the blank standard was run in duplicate in each plate. Lutein standard was obtained from Extrasynthèse (Genay, France). Solvents used for extraction and colorimetry contained 0.01% of 2,6-di-tert-butyl-p-cresol (Fluka Chemika, Buchs, Switzerland) as an antioxidant. Repeatability of optical density readings was assessed from the standards; mean intra-plate coefficient of variation ± s.e.m. was 2.28±0.45% and mean inter-plate coefficient of variation ± s.e.m. was 2.77±0.53% (N=5 plates). Carotenoid concentration in the samples was determined using the mean curve of the ten standard curves. Repeatability of concentrations was assessed from the standards; mean intra-plate coefficient of variation ± s.e.m. was 3.78±0.95% and mean inter-plate coefficient of variation ± s.e.m. was 4.72±1.23%.

Statistical analysis

All statistical analyses were conducted using SAS v.8.2 (SAS Institute 1999–2001, Cary, NC, USA). Tests of residuals for normality (Shapiro–Wilk) and homoscedasticity were used to check the validity of the model. Repeated-measures analysis of variance (ANOVA) was used to test for differences in changes in wing web thickness, body mass and plasma carotenoids between immune-challenged and control birds over the course of the experiment, i.e. on the seven capture occasions. Repeated-measures analysis of covariance (ANCOVA) was also used to investigate the sources of variation in immune response on day 1, using wing web thickness on days 0 and 1 as dependent variables. In the same way, variation in the pattern of decrease in immune response was analysed with repeated-measures ANCOVA, using wing web thickness on days 1, 2 and 4 as dependent variables. When investigating variation in wing web thickness with repeated-measure models, we specified a contrast with the first (initial or earliest) measurement in order to control for initial wing web thickness. Maximum variation in plasma carotenoid concentration between day 0 and day 2 was calculated as the minimum value attained on day 1 or day 2 minus the initial value on day 0. Preliminary analyses showed no significant aviary effect and these were therefore not included in the models. Tarsus length was first entered as a covariate in all models to control for any confounding effect of body size. Tarsus length was never statistically significant and was thus not retained in the final versions of the analyses. Values are given as means ± s.e.m.

RESULTS

Dynamics of the immune response

Injection of PHA resulted in a wing web swelling that peaked 24 h after injection (Fig. 1A). This difference in wing web thickness between PHA-injected and control birds stayed significant until day 4 and disappeared from day 7 (Table 1; Fig. 1A). The dynamics of the immune response and its duration varied among individuals, as revealed by the examination of individual response patterns (Fig. 2). Most individuals reached the peak in wing web swelling by day 1, except two birds (6%) that reached their peak by day 2, i.e. 48 h after injection. Among the birds showing a peak in wing web thickness after 24 h, 22 birds (67%) showed a rapid decrease in wing web swelling between days 1 and 2 whereas nine birds (27%) showed a slow decrease in wing web thickness between days 1 and 4. In the following, we investigated sources of variation in peak immune response and pattern of decrease in wing web swelling over the next three days, restraining the analyses to birds showing a peak on day 1.

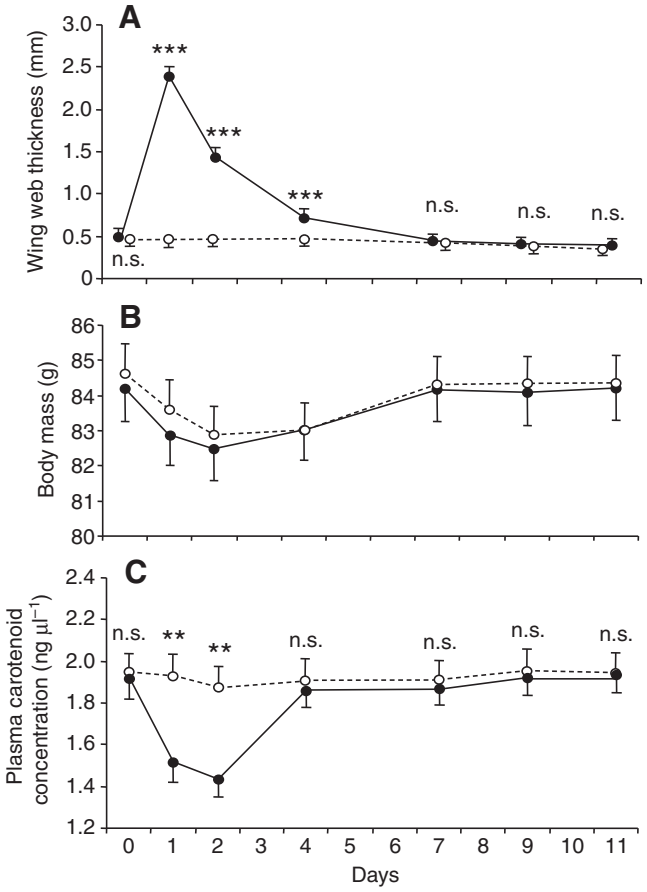

Fig. 1. Mean (±s.e.m.) variation over the course of the experiment in (A) wing web thickness (mm) (B) body mass (g) and (C) plasma carotenoid concentration (ng µl^{-1}) in immune-challenged (black circles, solid lines) and control (open circles, broken lines) blackbirds. Statistically significant differences between groups at P<0.001 and P=0.01 are indicated by *** and **, respectively. Non-significant differences are indicated by n.s. See Table 1 for corresponding statistics.

Body mass

All birds showed a transient mass loss during the course of the experiment, decreasing after injection until day 2 then increasing back to initial levels by day 7 (Fig. 1B). Compared with day 0, birds weigh significantly less on days 1, 2 and 4 (all contrasts with day 0: $F_{1,49}$>12.91, P<0.001) but not on day 7 (contrast with day 0: $F_{1,49}$=0.14, P=0.71). This variation in body mass was similar in PHA-injected and control birds (Table 1).

Table 1. Repeated-measures analyses of variance (ANOVA) for differences in cell-mediated immune response (wing web swelling), body mass and plasma carotenoids between immune-challenged (PHA-injected) and control (PBS-injected) birds

	Immune response		Body mass		Plasma carotenoids	
	$F_{d.f.}$	P	$F_{d.f.}$	P	$F_{d.f.}$	P
Multivariate tests						
Time	$51.91_{6,44}$	**<0.0001**	$12.39_{6,44}$	**<0.0001**	$4.10_{6,44}$	**0.002**
Time × injection	$47.66_{6,44}$	**<0.0001**	$0.69_{6,44}$	0.66	$2.16_{6,44}$	0.06
Univariate tests						
Day 0 (before injection)	$2.69_{1,49}$	0.11	$0.64_{1,49}$	0.43	$0.29_{1,49}$	0.59
Day 1 (24 h after injection)	$194.43_{1,49}$	**<0.0001**	$1.00_{1,49}$	0.32	$6.25_{1,49}$	**0.01**
Day 2	$23.16_{1,49}$	**<0.0001**	$0.62_{1,49}$	0.43	$6.55_{1,49}$	**0.01**
Day 4	$14.07_{1,49}$	**0.0005**	$0.28_{1,49}$	0.60	$1.05_{1,49}$	0.31
Day 7	$0.92_{1,49}$	0.34	$0.40_{1,49}$	0.53	$0.17_{1,49}$	0.68
Day 9	$0.23_{1,49}$	0.63	$0.52_{1,49}$	0.48	$1.05_{1,49}$	0.31
Day 11	$1.24_{1,49}$	0.27	$0.43_{1,49}$	0.52	$0.36_{1,49}$	0.55

Multivariate tests refer to Wilks' Lambda Manova test of the hypotheses of no effect of time and of no difference in dynamics between immune-challenged and control birds, i.e. no effect of the interaction time × injection. PHA, phytohemagglutinin; PBS, phosphate-buffered saline.

Immune response on day 1 was not significantly related to initial body mass (repeated-measures ANCOVA, interaction time × initial body mass, $F_{1,29}$=0.04, P=0.84).

The decrease in swelling between days 1 and 4 was not significantly related to initial body mass (repeated-measures ANCOVA, interaction time × initial body mass, $F_{2,28}$=1.80, P=0.18).

Plasma carotenoids

Immune-challenged birds showed a significant decrease in plasma carotenoid concentration compared with control birds on days 1 and 2 (Table 1; Fig. 1C). Plasma carotenoid levels of immune-challenged birds increased back to initial levels from day 2 to day 4, when they were not significantly different from that of control birds anymore (Table 1; Fig. 1C). Among PHA-injected birds, mean (±s.e.m.) maximum change in circulating carotenoids was –34.2±4.3% of the initial carotenoid levels (range –96.0% to +18.6%). The maximum change in plasma carotenoid concentration between day 0 and day 2 was negatively related to initial carotenoid concentration; the higher the initial carotenoid levels on day 0, the greater the decrease in plasma carotenoid concentration (Fig. 3). An index of relative maximum change in carotenoid concentration independent of initial level was calculated as the residuals of this regression. We used this index to test whether changes in circulating carotenoids were associated with the immune response.

Immune response on day 1 was significantly negatively related to initial plasma carotenoids levels (repeated-measures ANCOVA, interaction time × initial plasma carotenoids, $F_{1,28}$=11.18, P=0.002; univariate analysis of wing web thickness on day 2: $F_{1,28}$=13.07, P=0.001, slope estimate ± s.e.m. = –0.31±0.09) (Fig. 4A). In the same model, the relative change in plasma carotenoids between days 0 and 2 also significantly explained immune response on day 1. Individuals that showed a relatively greater decrease in plasma carotenoids developed a more important swelling (repeated-measures ANCOVA, interaction time × relative change in plasma carotenoids, $F_{1,28}$=4.90, P=0.03; univariate analysis of wing web thickness on day 2: $F_{1,28}$=7.15, P=0.01, slope estimate ± s.e.m. = –0.44±0.16) (Fig. 4B). Initial plasma carotenoids and its relative change together explained 41.4% of the variation in wing web thickness on day 1.

The decrease in wing web swelling between days 1 and 4 was significantly influenced by initial plasma carotenoid levels (repeated-measures ANCOVA, interaction time × initial plasma carotenoids, $F_{2,27}$=6.65, P=0.004). In the same model, maintenance of the swelling was not significantly related to the relative change in plasma carotenoids (repeated-measures ANCOVA, interaction time × relative change in plasma carotenoids, $F_{2,27}$=2.62, P=0.09). From Fig. 2, it appeared that most of the difference between birds with slow and fast decreasing swelling thickness occurred between days 1 and 2. We therefore tested whether initial carotenoid levels had an effect on the decrease in wing web swelling between days 1 and 2, between days 2 and 4 or both, i.e. during the whole decrease phase. Between days 1 and 2, initial plasma carotenoids concentration had no significant effect on how quickly the swelling decreased (repeated-measures ANCOVA, interaction time × initial plasma carotenoids, $F_{1,28}$=0.49, P=0.49) (Fig. 5A). However, between days 2 and 4, the decrease in swelling was significantly slower in individuals with high initial plasma carotenoids levels (repeated-measures ANCOVA, interaction time × initial plasma carotenoids, $F_{1,28}$=4.96, P=0.03) (Fig. 5B).

Carotenoid-based bill colour

Bill colour decreased from day 0 to 11 (Wilcoxon sign rank test S=–285.5, P<0.0001) but this decrease was not stronger in immune-challenged birds than in control birds, as would be expected from

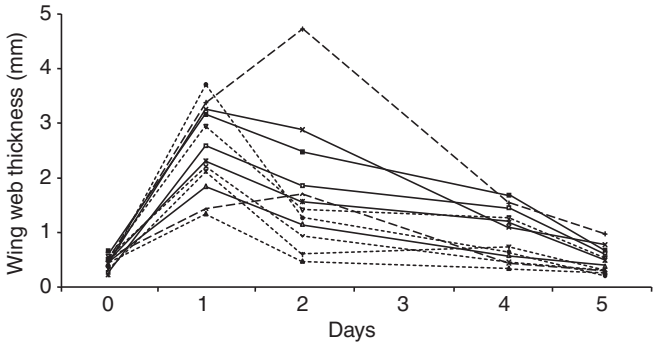

Fig. 2. Individual wing web thickness in immune-challenged birds over time. For clarity, only 12 individuals are presented; the two birds showing a peak in wing web thickness on day 2 (thick broken line) and among the birds showing a peak on day 1, five of those showing a slow (solid line) or rapid (thin broken line) decrease in wing web thickness between days 1 and 4.

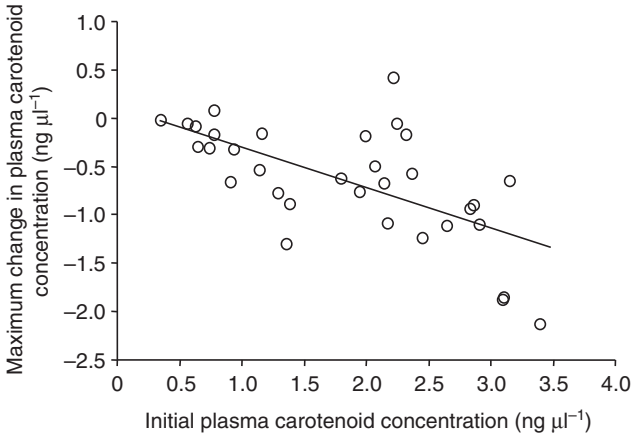

Fig. 3. Maximum change in plasma carotenoid concentration (ng µl^{-1}) between days 0 and 2 in immune-challenged birds, as a function of initial value on day 0 ($F_{1,31}$=21.49, P<0.0001, slope estimate ± s.e.m. = –0.42±0.09).

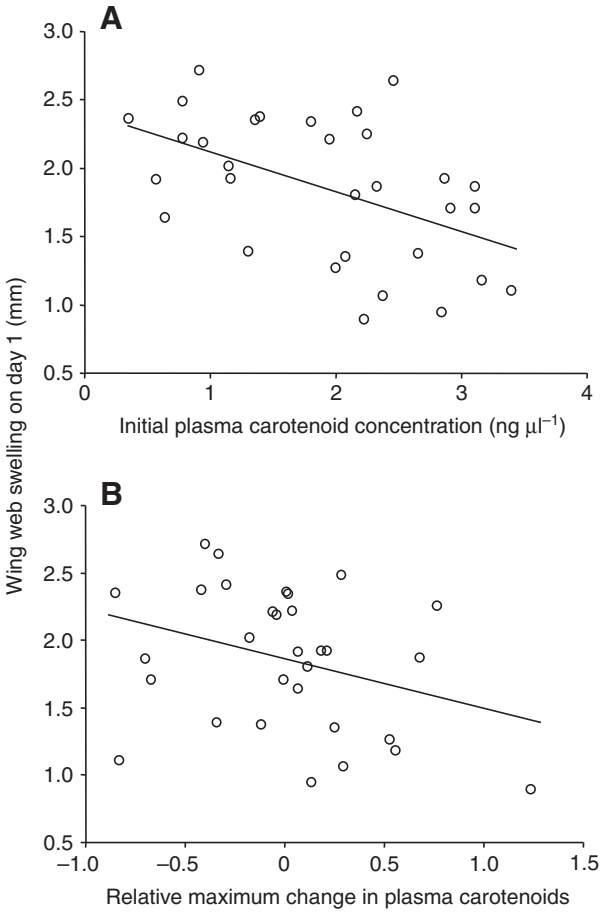

Fig. 4. Wing web swelling on day 1 (mm), calculated as the difference in thickness between days 1 and 0 as a function of (A) initial plasma carotenoids concentration (ng µl^{-1}) and (B) the relative maximum decrease in plasma carotenoids (negative residuals indicate a more important decrease and positive values a less important decrease than expected from the individual's initial plasma carotenoid concentration, see Fig. 3 and text for details).

the prediction that bill colour would reflect changes in plasma carotenoids (one-tailed Wilcoxon two-sample test, Z=1.33, P=0.09) (Fig. 6). Initial bill colour was significantly positively correlated to plasma carotenoid levels on day 0 ($r_{Spearman}$=0.30, P=0.03). Initial bill colour however did not significantly predict wing web swelling on day 1 (repeated-measures ANCOVA, interaction time × initial colour score, $F_{1,31}$=0.42, P=0.52) or its decrease between days 1 and 4 (repeated-measures ANCOVA, interaction time × initial colour score, $F_{2,30}$=0.38, P=0.69).

DISCUSSION

Although PHA-induced immune response is one of the most widely used immune challenge in immuno-ecology studies in vertebrates, particularly in birds, we are only beginning to investigate its detailed physiological mechanisms in relation to its potential costs in wild birds (Martin et al., 2006; Hõrak et al., 2007; Pérez-Rodríguez et al., 2008; Tella et al., 2008). In this study, we investigated the temporal dynamics of PHA-induced immune response and potential associated changes in blood carotenoids, body mass and a carotenoid-based coloured signal.

The peak of the immune response to PHA was reached within 24 h after injection, except in two cases (see also Ewenson et al., 2003; Martin et al., 2003; Navarro et al., 2003). Two contrasting temporal patterns in the subsequent decrease in wing web swelling were detected. The majority of birds showed a steep decrease back to initial thickness within the next 24 h, while for *ca*. 30% of birds, wing web thickness decreased slowly until day 7, suggesting that in this last case immune response and/or inflammation at the site of injection was maintained for longer. Wing web swelling after PHA injection has indeed been found to reflect the local inflammatory response (Martin et al., 2006; Tella et al., 2008) as well as the activation of the T-cell-mediated immune system through changes in the amount of circulating T-lymphocyte subsets (Tella et al., 2008). To our knowledge, such individual variation in the duration of the swelling has not been studied previously, although it is relevant to the issue of immunocompetence and associated trade-offs. However, Navarro and colleagues (Navarro et al., 2003) investigated individual variation in latency to maximum immune response in the house sparrow and showed that stronger immune responses took longer to develop. They focused on the first 72 h after injection during which responses remained relatively stable after the peak was reached. An immune response maintained for longer might be adaptive if more efficient but might also be more costly in terms of energy or self-damage (Schmid-Hempel, 2003). Alternatively, it might not be adaptive and might result from a deficient control of the immune system, for example, in the case of unrestrained inflammatory activity. The type of immune response developed by an individual, i.e. fast or long-lasting, may be linked to the current trade-offs it faces [e.g. reproductive state and moult (Greenman et al., 2005); hormonal status (Deviche and Cortez, 2005)], and/or to constraints linked to the development of the immune system and environmental effects (see Martin et al., 2004). Understanding the proximate causes of individual variation in immune response patterns would probably be insightful if we are to interpret immune response to PHA in terms of immunocompetence and resistance to parasites. We therefore attempted to explain both peak intensity and decrease pattern of the swelling in relation to body mass, as an index of general body condition, and plasma carotenoids because of their role as immunostimulants and in the antioxidant system.

All birds experienced a transient body mass loss over the course of the experiment. This variation in body mass probably reflected

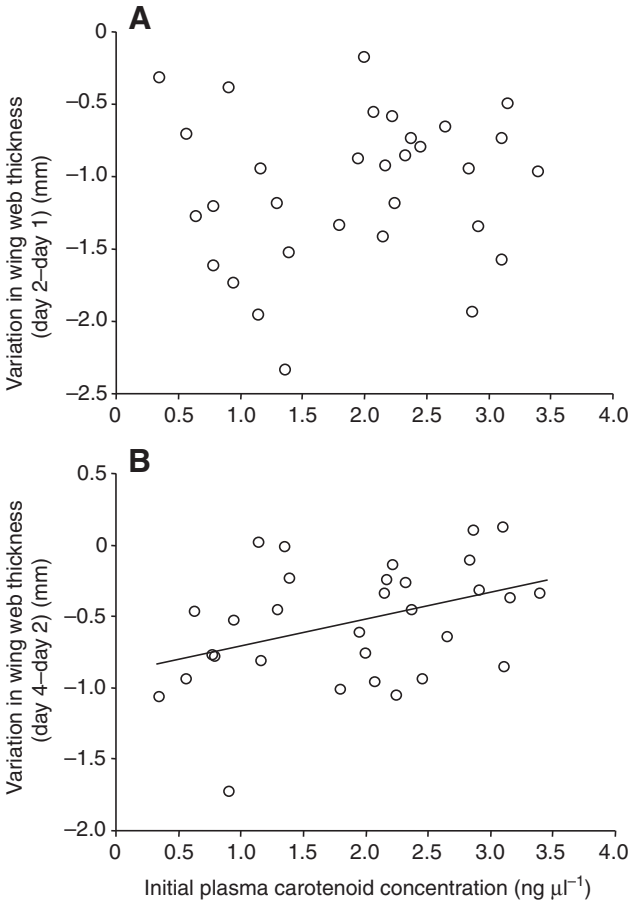

Fig. 5. Decrease in wing web swelling (mm) (calculated as swelling on one day minus swelling on the previous day) as a function of initial plasma carotenoid concentration (ng μl^{-1}) between (A) days 1 and 2 and (B) days 2 and 4 (slope estimate ± s.e.m. = 0.18±0.08).

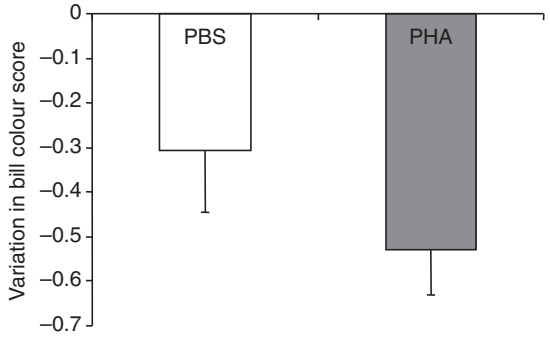

Fig. 6. Mean (±s.e.m.) variation in bill colour score between days 0 and 11 as a function of treatment group. PBS, phosphate-buffered saline; PHA, phytohemagglutinin.

the stress of the first captures and handling. However, the birds relatively quickly overcame this stress as body mass increased again between days 4 and 7 back to initial values. The absence of a significant difference between PHA and control birds suggests that the immune challenge did not cause any detectable energetic stress [consistent with Martin et al. and Greenman et al. (Martin et al., 2003; Greenman et al., 2005)]. Initial body mass did not influence the intensity of the immune response, which seems to be generally the case in adult birds (Alonso-Alvarez and Tella, 2001; Martin et al., 2003; Haussmann et al., 2005; Tella et al., 2008) (but see Navarro et al., 2003), or the pattern of decrease in wing web swelling. The birds were maintained for several months in captivity before this experiment and were provided with food *ad libidum*. Differences in body condition among birds may therefore have been too small to induce any differences in immune response (Alonso-Alvarez and Tella, 2001) and, conversely, PHA-induced immune response was not costly in terms of body mass.

Plasma carotenoid levels decreased in immune-challenged birds and this decrease followed a pattern that closely matched that of wing web swelling. This is the first study showing such inversely matched dynamics of circulating carotenoids with that of the immune response, indicative of a clear causal relationship. The two previous studies monitoring plasma carotenoid levels after PHA injection in captive birds reported contrasting results (Pérez-Rodríguez et al., 2008; Hõrak et al., 2007). A decrease in plasma carotenoids, although proportionally less important, was also observed in red-legged partridges *Alectoris rufa* one day after injection (Pérez-Rodríguez et al., 2008). No change in plasma carotenoids was detected in greenfinches *Carduelis chloris* three days after injection (Hõrak et al., 2007) at a time when, in our experiment, circulating carotenoids were increasing back to their initial level again. This suggests that in order to draw reliable conclusions on variation in carotenoid levels following immune challenge we should explore a temporal window rather than relying on single measurements.

Contrary to our expectations, there was a negative effect of initial plasma carotenoid concentration on the magnitude (peak) of the immune response; wing web swelling on day 1 was less important in individuals with high plasma carotenoid levels before injection (Fig. 4A). However, previous studies on the relationship between basal carotenoid levels and the intensity of the PHA-induced immune response reported different results; no relationship was found in greenfinches (Hõrak et al., 2007) and a positive result was found in partridges (Pérez-Rodríguez et al., 2008) (see also Blas et al., 2006). These mixed results might suggest either a species-dependent or condition-dependent effect of carotenoids on immune response or differences in food nutritional quality among studies. Plasma carotenoid levels may exert a dose-dependent effect on the immune response, which might vary according to species and dietary availability of carotenoids in natural conditions.

By contrast, we found a positive effect of the relative decrease in plasma carotenoids on the strength of the immune response. To our knowledge, only one previous study explicitly tested for a relationship between a change in plasma carotenoids and the intensity of PHA-induced immune response; in zebra finches *Taeniopygia guttata*, carotenoid levels decreased in experimental, testosterone-implanted birds, mounting a stronger immune response whereas this relationship was reversed in control birds (McGraw and Ardia, 2007). Likewise, in our study, individuals whose plasma carotenoid levels decreased relatively more after injection showed a greater wing web swelling on day 1. It still remains to be determined however whether this relationship is direct or indirect, i.e. whether carotenoids were involved in the development of the immune response or in other physiological processes activated as a consequence of the immune response. The capacity to mobilise plasma carotenoids may positively affect the magnitude of the immune response or a strong immune response may induce a more important decrease in plasma carotenoids. These two hypotheses linking variation in plasma carotenoids and the immune response in a direct and indirect way are not mutually exclusive. Carotenoids

exert important immunomodulatory functions that might be especially important in a PHA response, such as stimulating the production of cytokines and the activity of immune cells (reviewed by Chew, 1993; Møller et al., 2000), as well as regulating inflammation (Rafi and Shafaie, 2007; Kim et al., 2008). Changes in plasma carotenoid levels might therefore directly reflect their rapid re-allocation to immune tissues or cells (Koutsos et al., 2003). Carotenoids are part of the integrated antioxidant system in birds (Surai, 2002; Ewen et al., 2006; Costantini, 2008). Indeed, although the relative contribution of carotenoids to total antioxidant activity has been recently shown to be probably low and might vary among species (Costantini and Møller, 2008), carotenoids are involved in the antioxidant system of birds in many ways, not necessarily implying a direct contribution to antioxidant capacity (e.g. Costantini, 2008). For example, carotenoids exert a protective and recycling role for antioxidants like vitamins E and A (e.g. Edge et al., 1997; Surai and Speake, 1998; Surai et al., 2001; Surai, 2002). Changes in carotenoid levels might therefore directly or indirectly reflect short-term changes in oxidative status (Pérez-Rodríguez et al., 2008) due to the immune response that induces an increase in oxidative stress and associated damages (Costantini and Dell'Omo, 2006; Hõrak et al., 2007) and may also increase metabolic rate (Martin et al., 2003; Lee et al., 2005; Nilsson et al., 2007).

Individual differences in the maintenance of the skin swelling could also be partly explained by plasma carotenoids. The relative decrease in plasma carotenoids during the early immune response did not affect swelling duration. However, high initial carotenoid levels were associated with a slower decrease in wing web swelling in the late part of the PHA skin response: between days 2 and 4. Previous studies investigating the variation in physiological parameters associated with the immune response to PHA have yielded complex results on changes in oxidative stress, antioxidant status and lipid peroxidation and on when such changes occur during the response (Costantini and Dell'Omo, 2006; Hõrak et al., 2007; Pérez-Rodríguez et al., 2008). Plasma carotenoids, antioxidant capacity, oxidative stress and consecutive damages might influence the immune and inflammatory responses and, in particular, their dynamics might be linked. It would therefore be insightful to examine their potential simultaneous influence on the pattern of decrease of the swelling.

There was thus a contradictory dual effect of carotenoids. On the one hand, high plasma levels were detrimental to the development of the immune response. On the other hand, the decrease in circulating carotenoids was positively related to the strength of the immune response, and high initial carotenoid levels were associated with a slower decrease of the swelling. This suggests that carotenoid levels might have a complex effect on the immune and/or inflammatory response. More detailed investigations are thus needed to improve our understanding of the role of carotenoids in PHA-induced immune response and to assess how an allocation trade-off between immune function and carotenoid-based colour might ensure honesty in these signals.

The immune challenge did not induce a decrease in bill colour, suggesting that the immune response due to PHA was not sufficiently intense or long lasting to induce the re-allocation of carotenoids from the bill to circulation as suggested for humoral immune response (Faivre et al., 2003a). Although bill colour was correlated to initial plasma carotenoid levels (see also McGraw and Ardia, 2003), it did not predict the immune response either in its magnitude [contrary to Faivre et al. (Faivre et al., 2003b)] or duration. This suggests that bill colour reflects medium- or long-term quality rather than immediate quality. Accordingly, in the partridge, only the carotenoid-based colour of soft skin tissues was positively correlated to the strength of PHA response and reflected short-term changes in body condition and not that of the more keratinised bill (Pérez-Rodríguez et al., 2008; Pérez-Rodríguez and Viñuela, 2008).

In this study, we showed that individuals might strongly differ in the dynamics of the maintenance of the swelling response to PHA. Plasma carotenoids were closely associated with the development of the immune response and partly explained duration of the swelling. We could not explain the main variation in the decrease pattern in swelling observed here (i.e. within 24 h of peak response) with the variables we measured in this experiment. However, we would like to emphasise that interpreting PHA-induced immune responses in terms of immunocompetence would certainly gain from taking into account individual variation in response patterns as well as the proximate causes and consequences of these variations. The simultaneous assessment of antioxidant capacity and of oxidative-stress-induced damages has been underlined as a necessary approach to improve our understanding of the costs of immune reactions (Hõrak et al., 2007). We would suggest taking into account the dynamics of physiological parameters linked to, or influenced by, the immune response as important. Future studies in captive birds should ideally combine measuring several physiological parameters potentially associated with the immune response to that of its detailed dynamics.

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