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RESEARCH ARTICLE

Carotenoid intake does not affect immune-stimulated oxidative burst in greenfinches

Elin Sild, Tuul Sepp, Marju Männiste and Peeter Hõrak*

Department of Zoology, Institute of Ecology and Earth Sciences, The Centre of Excellence FIBIR, Tartu University, Vanemuise 46, 51014 Tartu, Estonia

*Author for correspondence (horak@ut.ee)

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SUMMARY

Carotenoid-based integument colouration is extremely widespread in the animal kingdom. It has been hypothesized that carotenoid colouration is used for communicating the health status of the bearers because carotenoids are efficient immunomodulators or antioxidants. However, the latter argument has been recently debated and the mechanisms by which carotenoids modulate immunity or oxidative balance are poorly known. We performed an experiment on wild-caught captive greenfinches, passerine birds with carotenoid-based plumage colouration, in order to test whether dietary carotenoid supplementation affects immune-stimulated oxidative burst of phagocytes in the whole blood and humoral immune response to a novel antigen, *Brucella abortus* (BA). Additionally, we tested whether immune stimulation with bacterial lipopolysaccharide (LPS) affects blood carotenoid levels. We thus tested the effects of carotenoids on the oxidative burst of phagocytes under neutral conditions and during *in vivo* immune challenge. LPS injection depleted plasma carotenoids, indicating involvement of these phytochemicals in the immune response. However, we did not find any evidence that manipulation of carotenoid intake had modulated anti-BA antibody production, LPS-stimulated oxidative burst of phagocytes, or basal levels of circulating reactive oxygen species. This indicates that carotenoid intake does not affect endogenous production of reactive oxygen species by immune cells. This finding is consistent with the view that carotenoids are unlikely to provide a direct link between oxidative stress and colouration. However, it remains to be tested whether the oxidative burst of phagocytes induced in our experiment actually inflicts oxidative damage and whether carotenoids play a role in the attenuation of such potential damages.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/20/3467/DC1

Key words: *Brucella abortus*, carotenoid, immune challenge, LPS, passerine, whole blood chemiluminescence response.

INTRODUCTION

Carotenoid pigments are extensively used for the colouration of the integument of birds, fishes and reptiles. Although vertebrates cannot synthesise carotenoids *de novo*, these pigments are common components of the colour of signals used in mate attraction and other types of social communication (Blount and McGraw, 2008; Møller et al., 2000; Pérez-Rodríguez, 2009; Svensson and Wong, 2011). The question about what qualities animals communicate by these pigments rose into the focus of physiological ecologists after recognizing the potential health impact of carotenoids. The link between carotenoid-based ornamentation and immune function was first suggested by Lozano (Lozano, 1994) and later developed by von Schantz et al. (von Schantz et al., 1999), who emphasized the antioxidant function of carotenoids and highlighted the connection between ornaments, oxidative stress and immune function. Accordingly, carotenoid-based ornaments enable individuals to signal their past and/or current health state; carotenoids can be allocated to signalling only if and when they are not needed for other purposes at the same time. Recently, however, the direct connection between the antioxidant properties of carotenoids and colouration has been debated (Costantini and Møller, 2008; Hartley and Kennedy, 2004; Pérez-Rodríguez, 2009).

Experiments attempting to prove the health-promoting effects of carotenoids *via* immune function have yielded contradictory results (reviewed by Chew and Park, 2004; Krinsky and Johnson, 2005; McGraw et al., 2011; Sepp et al., 2011). One of the reasons for this inconsistency may lay in the tight interconnections between the immune and antioxidant systems and the potential ability of carotenoids to quench reactive oxygen species (ROS) (Svensson and Wong, 2011). ROS production *via* oxidative burst (also named respiratory burst) is the main effector mechanism of microbial killing by phagocytic cells. On contact with microbial antigens, these cells activate the NADPH oxidase system, which results in the production of superoxide anion (O_2^-) from molecular oxygen (e.g. Freitas et al., 2009). Superoxide interacts with other molecules to generate more noxious reactive species such as peroxynitrite and hydroxyl radicals and hypochlorous acid. These compounds exhibit a broad spectrum of biotoxicity and thus form a crucial component of innate immunity, serving as the first line of defence against microbial infection (reviewed by He et al., 2007; Palozza et al., 2010). However, prolonged production of high levels of ROS, as in chronic inflammations, can cause intense oxidative stress, leading to the accumulation of oxidative damage in tissues (e.g. Kielland et al., 2009).

Because of this dual role, ROS production by phagocytic cells poses a challenge for understanding the health-promoting action of carotenoids. If carotenoids are efficient antioxidants, then their excess may impair immune function by counteracting the ROS production required for pathogen destruction; in contrast, carotenoids may be required for protection of immune cells and proteins from the collateral damage induced by ROS. At present, some indirect evidence exists in support of the both scenarios. Probably the best example of

protection conferred by carotenoids against damage generated by phagocytes originates from the study of *Staphylococcus aureus*, where a mutant strain with disrupted carotenoid synthesis was less able to resist a neutrophil oxidative burst (Liu et al., 2005). Adding different carotenoids to mammalian inflammatory cell cultures can remarkably inhibit oxidative burst (Murakami et al., 2000; Siems et al., 2003; Walrand et al., 2005). Further, dietary carotenoid supplementation can impair the phagocytosis ability of avian blood (McGraw and Klasing, 2006) and suppress inflammation (Koutsos et al., 2003; Koutsos et al., 2006; Meriwether et al., 2010; Shanmugasundaram and Selvaraj, 2011). However, under some circumstances, dietary carotenoid supplementation can also enhance the bacterial killing capacity of blood (Chew and Park, 2004; McGraw et al., 2006; McGraw and Klasing, 2006; McGraw et al., 2011). It is possible that the pro-inflammatory *versus* anti-inflammatory function of carotenoids depends on the interaction between dietary carotenoids and fatty acids (e.g. Selvaraj et al., 2005).

Here we apply a novel approach for testing whether and how dietary carotenoids affect ROS production by avian blood. We use a Pholasin-based whole blood chemiluminescence (WBCL) assay, which measures an inducible component of innate immunity by quantifying ROS (primarily superoxide) production by phagocytes on stimulation by bacterial lipopolysacharide (Sild and Hõrak, 2010). Previous experiments with female greenfinches (*Carduelis chloris*) have demonstrated the biological importance of this component of oxidative burst, as the WBCL response was sensitive to *in vivo* priming with *Escherichia coli* lipopolysaccharide (LPS) and *Brucella abortus* (BA) antigen suspension (Sild and Hõrak, 2010). Furthermore, WBCL response in greenfinches consistently covaries with behavioural traits such as captivity tolerance (Sild et al., 2011). Our study objects are greenfinches, sexually dichromatic seed-eating passerines that display a variety of carotenoid-based plumage patches, ranging from bright yellow to olive. The colour of the contrasting yellow tail patch is sensitive to infections, reflects immune responsiveness to some foreign antigens and is strongly associated with dietary and plasma lutein concentration and general physiological condition (reviewed by Aguilera and Amat, 2007; Hõrak et al., 2010; Peters et al., 2008). We supplemented half of 60 male greenfinches with dietary carotenoids in physiological dose and asked whether this affects the parameters of WBCL response. On the basis of published evidence, we had no predictions about the direction of the response. For instance, if the pro-inflammatory *versus* anti-inflammatory action of carotenoids depends on acquisition of dietary lipids (and hence body condition), it might imply that carotenoids stimulate oxidative burst in some individuals, whereas other birds could instead exhibit an inhibition, leading to an overall non-significant effect of the carotenoid treatment. We therefore tested the effect of carotenoid intake upon WBCL response under two different circumstances: under neutral conditions and during *in vivo* immune challenge. In the latter case, all the birds were vaccinated with a BA antigen, which induces a Th1-cell mediated, i.e. inflammatory, immune response (Khatun et al., 2009). Additionally, half of the birds in both carotenoid-supplemented and unsupplemented groups received an injection of another inflammatory agent, *E. coli* LPS. We expected that, under such challenging conditions, the possible effects of carotenoid intake upon oxidative burst of phagocytes might be different than under benign conditions where most of experiments with captive animals are conducted.

Vaccination with BA antigen enabled us to measure the strength of antibody response against BA. A previous study of another greenfinch subspecies has shown that dietary lutein supplementation increased anti-BA antibody production (Aguilera and Amat, 2007); we were thus interested in whether we could reproduce this result. We also asked whether induction of an inflammatory response *in vivo* depletes plasma carotenoids as demonstrated in some experiments on birds (Alonso-Alvarez et al., 2004; Koutsos et al., 2003; McGraw and Klasing, 2006; Biard et al., 2009; Shanmugasundaram and Selvaraj, 2011).

MATERIALS AND METHODS

Study protocol

Sixty male wild greenfinches, *Carduelis chloris* (Linnaeus 1758), were captured in mist nets at bird feeders in a garden in the city of Tartu (58°22′N, 26°43′E) on 4–8 January 2010. The birds were housed indoors in individual cages (27×51×55 cm) with sand-covered floors. Mean (±s.d.) temperature in the aviary during the experiment was 12.7±1.9°C and mean humidity was 48.0±8.6%. The birds were supplied *ad libitum* with sunflower seeds and tap water. Birds were held on the natural day-length cycle on artificial lighting, increasing continuously the length of the light period from 8 to 13 h by the end of the study. The birds were released into their natural habitat on 11 March. The study was conducted under the license from the Estonian Ministry of the Environment and the experiments comply with the current laws of the Estonian Republic.

The time course of the experiment is shown in Fig. 1. From 11 to 14 January, all of the birds were subjected to 4 day anticoccidian treatment with Intracox Oral (Interchemie, Castenary, The Netherlands) in order to reduce infection-induced variation in the physiological condition of individuals. The birds received 2 ml l^{-1} of the solution containing 25 mg l^{-1} Toltrazuril in their drinking water. Birds were divided into two groups with similar mean body mass on 14 January (30.97±1.84 g in supplemented birds *versus* 30.24±1.85 g in unsupplemented birds; t=1.5, P=0.134) and age composition (13 yearlings and 17 older birds in both groups). From 16 January, half of the birds started receiving 10 μg ml^{-1} carotenoid solution in their drinking water. Carotenoid supplementation consisted of lutein and zeaxanthin (20:1, w/w), prepared from an OroGlo liquid solution of 11 g kg^{-1} xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium). Lutein, followed by zeaxanthin, is the main carotenoid in the plasma of greenfinches, and both are converted to canary xanthophylls A and B in the integument (Saks et al., 2003; McGraw, 2004). Solutions were freshly prepared each evening using filtered (Brita® Classic; BRITA GmbH, Taunusstein, Germany) tap water and were provided in 50 ml doses in opaque dispensers in order to avoid oxidation of carotenoids. Carotenoid dose was determined on the basis of a previous study (Hõrak et al., 2006), in which birds that were supplemented daily with the same dose of lutein circulated approximately 30 μg ml^{-1} carotenoids after 23 days. According to Hõrak et al. (Hõrak et al., 2006), plasma carotenoid levels of wild greenfinches in January ranged from 1.5 to 21.6 μg ml^{-1}, averaging 10.2±6.6 μg ml^{-1} (N=13). After 15 days spent in captivity, these values decreased to 3.8±2.7 μg ml^{-1} in unsupplemented birds.

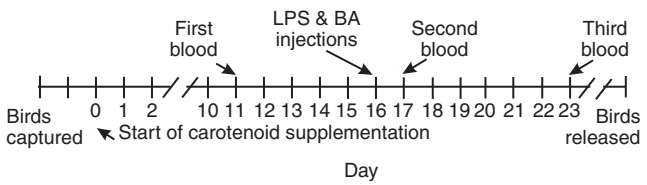

Fig. 1. Time course of the experiment. Day 0=16 January. BA, *Brucella abortus*; LPS, lipopolysaccharide.

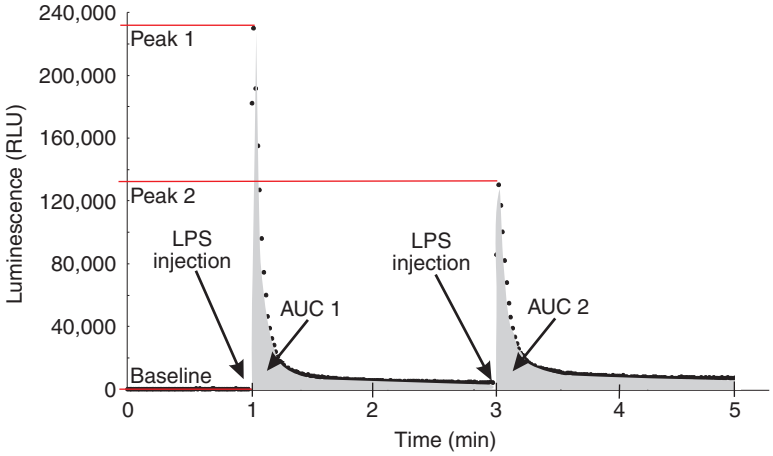

Fig. 2. Time course and parameters of the whole blood chemiluminescence (WBCL) response. Each circle corresponds to the measurement of luminescence at 1 s intervals. The measurement of baseline luminescence started after the plate had been incubated in the spectrophotometer for 1 min and lasts for 1 min. Thereafter, 25 µl of LPS solution was injected, followed by measurement of LPS-induced luminescence for 2 min. Peak 1, maximum luminescence occurring after the first LPS injection; AUC 1, the corresponding area under the curve (grey area between 1 and 3 min). At 3 min, the LPS injection was repeated and corresponding Peak 2 and AUC 2 were measured. RLU, relative luminescence units.

Birds were blood sampled on 27 January (i.e. 11 full days since the start of dietary treatment) and on 2 February (i.e. 17 full days since the start of dietary treatment) for assessment of the innate immune function on the basis of the oxidative burst of phagocytes (see below). On 1 February, all the birds were injected with 40 µl of killed BA suspension in the pectoralis muscle (serum agglutination test antigen, Strain 99, product code RAA0054, Veterinary Laboratories Agency, Weibridge, UK). Birds were blood sampled to determine antibody response to BA 6 days later. Simultaneously with BA injection, 16 birds in the carotenoid-supplemented group and 14 birds in the unsupplemented group received an injection of 0.01 mg *E. coli* LPS in 40 µl sterile isotonic saline in the pectoralis muscle (the same strain that was used later for induction of the oxidative burst in the blood). The rest of the birds received injections of 40 µl isotonic saline.

The total amount of blood extracted was approximately 200 µl on the first and second blood sampling and ca. 130 µl on the third blood sampling. Blood sampling took place in the mornings before the lights were turned on. Other procedures, including maintenance and BA injections, were performed in the evenings after the lights were turned off. For measurement of oxidative burst, blood from tarsal or brachial veins was collected into 200 µl Microvette® tubes (K3E) with EDTA tripotassium salt as an anticoagulant as described by Sild and Hõrak (Sild and Hõrak, 2010). Blood was maintained at room temperature until analysis during the same day. For measurement of antibody response to BA, blood was collected into tubes not containing anticoagulant, as to enable separation of serum.

Immune assays

Oxidative burst in the whole blood samples was measured using an ABEL® Cell Activation test kit with Pholasin® and Adjuvant-K™ (Knight Scientific, Plymouth, UK) according to the protocol of Sild and Hõrak (Sild and Hõrak, 2010). First, 20 µl of whole blood was diluted in 2 ml blood dilution buffer. Then, 85 µl of reconstitution and assay buffer, 20 µl of reconstituted Adjuvant-K, 50 µl of Pholasin and 20 µl of diluted whole blood were added to an opaque white 96-well microplate well. The process of measurement of the WBCL response as well as the measured variables are shown in Fig. 2. First, after inserting the plate into the spectrophotometer, it was incubated with medium shaking at 37°C for 1 min, and afterwards luminescence was measured for 1 min before the first LPS injection. Thereafter, 25 µl of LPS (1 mg ml^{-1} solution from *E. coli* serotype 055:B5; Sigma L2880) was injected with an automatic dispenser. Luminescence was recorded at 1 s intervals for 2 min; thereafter, another 25 µl dose of LPS was injected and luminescence was recorded for a further 2 min. Secondary injection of LPS induced another rapid peak in luminescence, which was usually higher than the first peak. We thus recorded seven different parameters of WBCL response: maximum luminescence after the first and second LPS injection (Peak 1 and Peak 2); the ratio of these peaks (height of Peak 2/height of Peak 1); the areas under the curve, measured for 2 min after the first and second LPS injections (AUC 1 and AUC 2, respectively); summed AUC (i.e. AUC 1 + AUC 2); and mean baseline luminescence, measured during 1 min before the first LPS injection (see Fig. 2 for graphical presentation of these parameters). Functionally, the heights of the first and second peaks describe the maximum capacity of phagocytes to produce ROS in response to stimulation. The first peak characterises the capability of oxidative burst of unprimed cells whereas the second peak is indicative of the capability of oxidative burst of (*in vitro*) primed cells. Peak ratio thus indicates the extent of potentiation of phagocytes (the higher the ratio, the higher the potentiation). Areas under the curve characterise the total amount of ROS produced in response to stimulation *in vitro*. The radical detected by such stimulation is (at least) predominantly superoxide. Measurement precision and further details of the assay are described by Sild and Hõrak (Sild and Hõrak, 2010).

Antibody response to BA was quantified according to the method of Amat et al. (Amat et al., 2006) and Munns and Lamont (Munns and Lamont, 1991) with minor modifications. Briefly, 45 µl of serum was added to 45 µl PBS (Sigma P-4417) in the first well of a 96-well microtitre plate and serial double dilutions in PBS (1:2, 1:4, 1:8 etc) were then carried out in the consecutive wells. Then, 45 µl of BA serum agglutination test antigen was added to each well. After incubation of plates for 24 h at room temperature, agglutination was visually scored. BA-positive (RAB1003) and -negative (RAB0701, Veterinary Laboratories Agency, Weybridge, UK) serums were used as controls. All samples were scored blindly by three different observers; the repeatability (Lessells and Boag, 1987) of agglutination scores was 0.91 ($F_{47,110}$=30.9, P<0.0001). The sample size for BA antibody titres was smaller than that used for assessment of oxidative burst because of our inability to collect sufficient amount of serum from all birds. The concentration of carotenoids was determined spectrophotometrically using a microplate reader and acetone-resistant microtitre plates. One hundred and fifty microlitres of acetone was added to 15 µl of plasma and centrifuged for 10 min at 1500 ***g***. Absorbance of supernatant was measured at 449 nm, corresponding to the maximum absorbance of lutein in acetone (Zsila et al., 2001). Calibration curves were prepared using lutein (Sigma X 6250) as a standard.

Table 1. Comparison of the parameters of the whole blood chemiluminescence (WBCL) response (in relative luminescence units, see Fig. 2), plasma carotenoids (μg ml^{-1}) and *Brucella abortus* (BA) antibody titres (maximum number of wells with agglutination in log 2 scale) in carotenoid-supplemented and unsupplemented (control) greenfinches

Days since the start of carotenoid supplementation	Parameter	Control		Carotenoid		*t*	*P*	Effect size (95% CI)
		Mean ± s.d.	*N*	Mean ± s.d.	*N*			
11	Plasma carotenoids	11.31±9.47	27	18.70±9.82	29	2.87	0.006	0.72 (0.22–1.31)
	Peak 1	128648±36036	30	122904±26576	30	0.70	0.49	0.18 (–0.33–0.69)
	Peak 2	177581±24876	30	167549±28146	30	1.46	0.30	0.37 (–0.14–0.89)
	Peak ratio	1.47±0.39	30	1.41±0.35	30	0.56	0.58	0.16 (–0.36–0.65)
	AUC 1	2157578±396310	30	2061711±426159	30	0.90	0.37	0.02 (–0.28–0.74)
	AUC 2	4775514±777628	30	4552067±877296	30	1.04	0.30	0.03 (–0.24–0.78)
	Sum AUC	6933092±1132501	30	6613778±1289016	30	1.02	0.31	0.26 (–0.25–0.77)
	Baseline	932±259	30	904±108	30	0.48	0.63	0.14 (–0.38–0.63)
17	Plasma carotenoids	6.57±6.01	29	14.02±6.87	30	4.43	<0.0001	1.00 (0.60–1.70)
	Peak 1	115126±29196	30	113577±37165	30	0.18	0.86	0.05 (–0.46–0.55)
	Peak 2	131986±36548	30	128129±38352	30	0.40	0.69	0.10 (–0.40–0.61)
	Peak ratio	1.20±0.41	30	1.19±0.41	30	0.06	0.95	0.02 (–0.49–0.53)
	AUC 1	1574728±319175	30	1563191±341711	30	0.14	0.89	0.00 (–0.47–0.54)
	AUC 2	2983534±1004257	30	2993529±977134	30	0.04	0.97	0.00 (–0.52–0.50)
	Sum AUC	4558262±1278406	30	4556720±1252917	30	0.00	1.00	0.00 (–0.51–0.51)
	Baseline	643±183	30	636±111	30	0.20	0.85	0.05 (–0.46–0.56)
23	BA titre	5.6±2.1	25	5.7±2.5	22	0.20	0.51	0.06 (–0.51–0.63)

Standardized effect size is the difference between means in standard deviation units. Effects of carotenoid supplementation on measured parameters did not became significant in ANCOVAs accounting for covariates such as initial trait values on day 11 or the time lag from blood sampling to analyzing, or for factors such as LPS treatment or age. Full models with all covariates and interaction terms between factors are presented in supplementary material Tables S1 and S2. AUC, area under curve.

Statistics

The effects of dietary carotenoid intake on immune parameters were analysed using analysis of covariance (ANCOVA), adjusting for the effects of the time lag from blood sampling to measurement (in case of the parameters of WBCL response), age (yearling *versus* older), LPS treatment, and initial trait values (in case of WBCL response parameters measured from the second blood). Full models, including interactions between factors, are presented in supplementary material Tables S1 and S2. Assumptions for parametric models (normality of residuals, homogeneity of variances) were met for all the models. Statistics from *t*-tests along with effect sizes for plasma carotenoid levels and values of immune parameters of carotenoid-supplemented and unsupplemented birds are presented in Table 1. All tests are two-tailed with α=0.05 as a criterion for significance. Means are presented ±s.d. unless otherwise indicated.

RESULTS

After 11 days of supplementation, carotenoid-fed greenfinches had, on average, 40% higher plasma carotenoid levels than unsupplemented birds. After 17 days, the difference was 53% (Table 1). LPS treatment reinforced the decrease in plasma carotenoid levels between the first and second blood sampling but carotenoid supplementation did not attenuate the decrease in circulating carotenoid levels due to LPS (Table 2, Fig. 3). We could not detect an effect of carotenoid supplementation on any parameters of the WBCL response (Table 1). Yearling greenfinches produced a stronger antibody response to BA than older birds (6.7±2.3, N=20 *vs* 4.8±1.9, N=27; $F_{1,42}$=9.3, P=0.004), but antibody titres were not affected by carotenoid supplementation in a model accounting for the effects of age ($F_{1,42}$=9.3, P=0.004), carotenoid treatment ($F_{1,42}$=0.4, P=0.512), LPS treatment ($F_{1,42}$=0.4, P=0.549) and carotenoid × LPS interaction ($F_{1,42}$=1.1, P=0.299).

DISCUSSION

Our experiment was successful in increasing plasma carotenoid levels by 40–53% in supplemented compared with unsupplemented birds. Plasma carotenoid concentrations of supplemented birds fell within the physiological range of wild greenfinches, whereas the mean carotenoid levels of unsupplemented birds, particularly on the second measurement event, were lower than previously reported for wild greenfinches [5.0–91.4 μg ml^{-1} (Aguilera and Amat, 2007); 4.6–21.3 μg ml^{-1} (Peters et al., 2008); 7.7–20.2 μg ml^{-1} (Sepp et al., 2010); 26.8±15.1 μg ml^{-1} (Tella et al., 2004)].

Why was the WBCL response of greenfinch blood not affected by carotenoids? Although we cannot totally rule out the possibility that we would have been able to detect an effect with a larger sample size, it should be noted that our sample size (2×30) exceeds that of most ecophysiological studies. Further, given that the effect of supplementation on plasma carotenoids was particularly strong (Table 1), we can be confident that induction of a 40–53% difference in carotenoid levels between treatment groups was insufficient for induction of significant differences between those groups in terms of WBCL response. Our results thus most likely reflect the genuine absence of a relationship between carotenoid access and phagocytes'

Table 2. Effects of carotenoid supplementation and LPS injection on plasma carotenoid levels of greenfinches after 17 days of supplementation

Effect	d.f.	*F*	η^2	*P*
Initial carotenoids	1, 50	59.5	0.54	<0.00001
Carotenoids	1, 50	11.9	0.19	0.001
LPS	1, 50	5.2	0.09	0.027
Carotenoids × LPS	1, 50	2.7	0.05	0.107

Initial carotenoids refers to the plasma carotenoid levels after 11 days of supplementation. η^2 is a coefficient of partial determination, describing the proportion of variance attributable to the predictor variable.

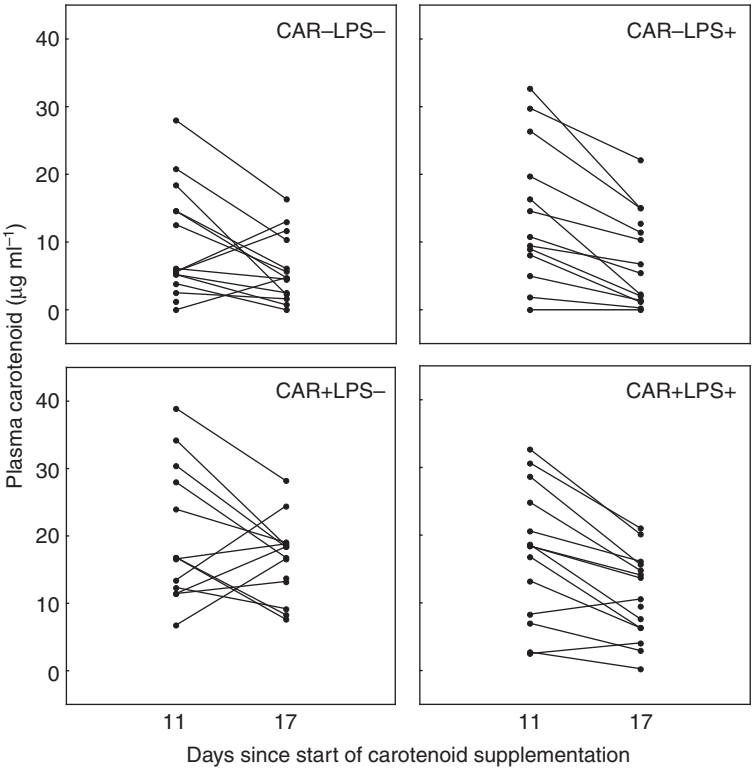

Fig. 3. Effects of carotenoid supplementation (CAR+) and LPS injection (LPS+) on the dynamics of individual plasma carotenoid levels of greenfinches. See Table 2 for *P*-values. Mean (±s.e.m.) values for change in plasma carotenoid levels ($\mu g\,ml^{-1}$) are as follows: CAR–LPS–: –4.20±1.77; CAR–LPS+: –6.45±1.77; CAR+LPS–: –3.52±1.84; and CAR+LPS+: –6.40±1.77.

capability for oxidative burst under our experimental conditions. This absence of a relationship is particularly noteworthy, given that we measured a number of parameters of ROS production, including the baseline levels, and that we tested the effects of carotenoid supplementation under different conditions. After 11 days of supplementation, the birds had not received any immune activation treatments and can be thus considered as experiencing relatively mild conditions. After 17 days of supplementation, all the birds had received a BA injection and half of them also received an *E. coli* LPS injection the previous night. Such treatments induce strong inflammatory responses (e.g. Owen-Ashley and Wingfield, 2007) and also increase the capability of phagocytes for ROS production, as shown in a pilot study of female greenfinches (Sild and Hõrak, 2010).

Only few previous studies have tested the effect of *in vivo* carotenoid administration on the capability of ROS production by phagocytes. Marin and colleagues (Marin et al., 2011) found that astaxanthin supplementation suppressed hydrogen peroxide (H_2O_2) production in diabetic rats under baseline conditions (compared with non-diabetic unsupplemented rats under baseline conditions), but not when neutrophils were stimulated with phorbol 12-myristate 13-acetate (PMA). No effect on superoxide or nitric oxide production was found. In healthy humans, limitation of dietary carotenoid (β-carotene, lycopene and lutein) intake to 25% of usual increased H_2O_2 production of PMA-activated neutrophils by 38%, whereas supplementation for 5 weeks restored basal H_2O_2 generation (Walrand et al., 2005). In the same study, H_2O_2 production by neutrophils was not affected by an *in vitro* supplementation with β-carotene or lycopene in non-depleted subjects, but a significant decrease in H_2O_2 content was observed in neutrophils from carotenoid-depleted subjects. In a study of painted dragons (*Ctenophorus pictus*), dietary carotenoid supplementation did not affect baseline ROS levels (including superoxide and H_2O_2) (Olsson et al., 2008); interestingly, the carotenoid-based skin colouration was also unaffected. The lack of effects on baseline ROS levels in lizards is comparable to our results regarding baseline WBCL response, measured before stimulation.

Our study differs from the above-mentioned ones in many aspects: unlike mammals, our study objects possess carotenoid-based ornaments; avian heterophils, unlike mammalian neutrophils, do not use myeloperoxidase (which yields a powerful oxidant hypochlorous acid) for microbial killing; we used whole blood instead of isolated cells; and our assay, unlike the ones using PMA as a stimulant, measured immediate extracellular production of ROS (Sild and Hõrak, 2010). In domestic chickens, which possess carotenoid-based integument colouration, the dietary fat and dietary lutein levels have been shown to modulate inducible nitric oxide synthetase (iNOS) activity of macrophages: when fatty acid exposure is low, lutein exposure increases iNOS mRNA abundance, but when fatty acid exposure is higher, lutein exposure reduces iNOS mRNA abundance (Selvaraj and Klasing, 2006). Similar responses also occurred *in vivo* (Selvaraj et al., 2005), and they depended on interaction of lutein and fatty acids in signal transduction through nuclear hormone receptors PPAR and RXR. These immunomodulatory effects are likely mediated *via* effects on oxidative stress and resulting changes in NFκB signalling or *via* direct effects on gene expression *via* the PPARγ–RXR pathway (Meriwether et al., 2010; Selvaraj and Klasing, 2006; Selvaraj et al., 2010). Our results are probably not directly comparable to the previously mentioned ones because in our study, the radical detected was (at least) predominantly superoxide. [During the adjustment of the assay protocol, we detected that incubation of the blood dilution with superoxide dismutase reduced the parameters of luminescence by approximately 70% (Sild and Hõrak, 2010). Currently, we are not aware of the contribution of other reactive species such as hydroxyl and ferryl radicals, nitric oxide or peroxidases (all of which are capable of

photostimulation of Pholasin) to the LPS-induced WBCL response. However, their potential contribution is clearly smaller than that of superoxide.]

Current results on WBCL responses are also not directly comparable with those of studies showing the effect of carotenoids on the microbicidal ability of blood (McGraw et al., 2011) or inflammatory responses (e.g. Koutsos et al., 2006; Koutsos et al., 2007). Although oxidative burst is an important component of microbial killing and inflammation, there are several other pathways through which carotenoids can affect immune responses independently of ROS scavenging. For instance, carotenoids can restore the redox status of tissues and protect the polyunsaturated fatty acids of cell membranes, regulate membrane fluidity and gap-junctional connections, increase or decrease expression of cell surface adhesion molecules, and induce or inhibit immune cell apoptosis, activation and proliferation (Chew and Park, 2004; Koutsos et al., 2007; Webb and Villamor, 2007).

Similarly to the WBCL response, we failed to detect an effect of carotenoid supplementation on anti-BA antibody response. Our results thus differ from those of a study in a greenfinch subspecies *Carduelis chloris aurantiiventris*, where lutein-supplemented males mounted a significantly stronger anti-BA response than unsupplemented birds (Aguilera and Amat, 2007). In that study, plasma carotenoid levels before BA injection were slightly higher ($21\,\mu g\,ml^{-1}$) than in the present study ($14\,\mu g\,ml^{-1}$), and unsupplemented birds also circulated more carotenoids ($10.5\,\mu g\,ml^{-1}$) than unsupplemented birds in our experiment ($6.6\,\mu g\,ml^{-1}$). The experiment was conducted in wintering birds kept in similar captive conditions approximately 1 month earlier than the current study and the sample sizes were smaller than ours. We thus have no explanation why the results of these two studies in the same bird species differ with respect to the effect of carotenoids on immune response to BA antigen.

Injection of LPS had a significant effect on the dynamics of individual plasma carotenoid levels between the first and second blood-sampling event (Table 2). During that period, plasma carotenoid levels of most of birds declined; however, this decline was much more prevalent among LPS-injected birds than among saline-injected controls (Fig. 3, Table 2). The general decline in plasma carotenoids (which also occurred among the birds receiving carotenoids) can be most likely explained by the inflammatory response induced by BA injection of all birds (Sild and Hõrak, 2010). LPS injection evidently contributed to this decline. Similar patterns of LPS-induced decline in plasma carotenoids have been demonstrated in domestic chickens (Koutsos et al., 2003; Meriwether et al., 2010; Shanmugasundaram and Selvaraj, 2011), red junglefowl (*Gallus gallus*) (McGraw and Klasing, 2006) and zebra finches (*Taeniopygia guttata*) (Alonso-Alvarez et al., 2004; Gautier et al., 2008). In contrast, Toomey et al. (Toomey et al., 2010) did not detect any effects of weekly LPS injections on plasma, liver or plumage carotenoids in house finches (*Carpodacus mexicanus*), although the accumulation of some specific carotenoids in the retina was affected. However, the carotenoids were sampled 7 days post injection in house finches, so the possibility for the transient drop of plasma carotenoids cannot be excluded (Toomey et al., 2010).

The results of the present study, along with published evidence (reviewed by Chew, 1993; Chew and Park, 2004; Biard et al., 2009), thus clearly support the involvement of carotenoids in the inflammatory response. Depletion of plasma carotenoids in response to LPS administration might occur for several reasons; for instance, carotenoids may be incorporated into lymphoid tissues where they act as immunomodulatory agents (Koutsos et al., 2003). However, it is also possible that changes in carotenoid metabolism during the acute phase response are an indirect result of alterations in lipid metabolism, without any carotenoid-specific regulation of tissue uptake (Koutsos et al., 2003). It has been also suggested that carotenoids could be depleted from the plasma as antioxidants because of the excess production of reactive species during the oxidative burst associated with the inflammatory response (e.g. Allen, 1997; von Schantz et al., 1999). However, the absence of the effect of carotenoids on the parameters of the WBCL response in this study is not consistent with this hypothesis. Altogether, these results indicate that interconnections between carotenoid-based colouration, immune function and oxidative stress are much more complicated than originally thought. Our findings may be consistent with the view that carotenoids are inefficient antioxidants *in vivo* and, therefore, are unlikely to provide a direct link between oxidative stress and colouration (Hartley and Kennedy, 2004) (see also Olsson et al., 2008). A limitation of the current study was that we did not assess whether the WBCL responses induced in our experiment actually inflict oxidative damage and whether carotenoids might play a role in attenuation of such potential damages. Further studies on these interconnections are thus required before the messages of carotenoid-based animal colouration can be understood.

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