

The carotenoid conundrum: improved nutrition boosts plasma carotenoid levels but not immune benefits of carotenoid supplementation

Anne Peters · Steffen Magdeburg · Kaspar Delhey

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Abstract Carotenoids are widely heralded as central to honest signaling due to their dual roles as pigments and antioxidants/immunostimulants. The aim of this study is to test if diet quality and carotenoids alone or in an interaction influence condition, carotenoid availability in plasma and immune responsiveness. Therefore, a diet experiment during the moult of great tits, *Parus major*, was performed. In a two-way design, we manipulated general quality (digestibility, protein and vitamin content) as well as carotenoid (lutein) content of semi-synthetic diets. Higher quality diet improved individual condition since birds had greater body mass, and to a lesser extent, higher hematocrit. In addition to the expected positive effect of carotenoid supplementation and individual lutein consumption on circulating lutein, there was a positive effect of enhanced diet quality on plasma carotenoid levels. Carotenoid supplementation, but not diet quality, improved the local inflammatory response and maintenance of body mass during a humoral immune reaction. The enhancement of circulating carotenoid levels by improved general quality of the diet or individual condition could provide a testable, mechanistic explanation for the variation in effects of carotenoid supplementation studies.

Keywords Honest signaling · Condition · Plumage coloration · Immuno-ecology

Introduction

Carotenoid-based ornaments are considered prime candidates for honest signals of individual quality. This is first and foremost because—unlike other integumentary pigments—carotenoids cannot be synthesized by animals but must be obtained through the diet (for review, see McGraw 2006). As a consequence, animals with superior foraging skills or competitive ability have better developed ornaments, and a number of pioneering studies have shown that carotenoid-based ornaments can thus indicate individual quality and foraging success (for review, see Hill 2006). However, carotenoids are not only pigments but can fulfil a variety of physiological roles related to self-maintenance (for review, see Perez-Rodriguez 2009). Consequently, it has been proposed (Lozano 1994) that animals not only face the task of obtaining carotenoids in their diet but also the trade-off of allocating carotenoids to pigmentary function for ornamental coloration versus using them in support of essential functions such as immune responses or to combat oxidative stress. Although this hypothesis generated a large body of research (for reviews, see Alonso-Alvarez et al. 2008; Olson and Owens 1998; Perez-Rodriguez 2009; Peters 2007), evidence for these proposed functions of carotenoids remains mixed (see, also, Vershinin 1999).

Carotenoids are large lipophilic molecules that due to their conjugated double-bond structure are able to neutralize damaging reactive oxygen molecules (Bendich 1993). Although antioxidant functions have been demonstrated in vitro, the relevance of carotenoids as antioxidants in vivo can currently be neither supported nor

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A. Peters (✉) · S. Magdeburg · K. Delhey
Behavioural Ecology of Sexual Signals Group,
Max Planck Institute for Ornithology,
Vogelwarte Radolfzell, Schlossallee 2,
78315 Radolfzell, Germany
e-mail: Anne.Peters@monash.edu

A. Peters
School of Biological Sciences, Monash University,
Clayton VIC 3168, Australia

rejected (for review, see Perez-Rodriguez 2009), and could be highly dependent on context, for example the presence of other antioxidants (Catoni et al. 2008a). Additionally, carotenoids can fulfil a number of vital physiological functions that result in immunostimulatory effects (for review, see Perez-Rodriguez 2009). For example, some studies on birds have shown that carotenoid supplementation may improve certain immune functions (Blount et al. 2003; McGraw and Ardia 2003), but others did not (Hörak et al. 2007; Smith et al. 2007). Likewise, some studies demonstrated that immune activation can reduce circulating carotenoid levels (Perez-Rodriguez et al. 2008; Peters et al. 2004), but not all (Hörak et al. 2006). Thus, although the evidence is mixed, carotenoid-based coloration could signal not only foraging efficiency but also oxidative or immune state.

Body levels of carotenoids will not only be determined by carotenoid acquisition in the diet, and carotenoid allocation to body functions, but also by carotenoid utilisation (Hill 2000), the ability to absorb and circulate carotenoids present in the diet. This may strongly depend on general condition or nutritional state since carotenoids are transported by blood lipoproteins that are costly to manufacture and difficult to maximally elevate for ornament expression (McGraw and Parker 2006). It has been known for a long time that carotenoid deposition in ornaments can depend on nutritional condition (Hill and Montgomerie 1994) and that this can be partly independent of carotenoid access (Hill 2000), but to what extent circulating carotenoid levels or physiological benefits of carotenoids could also directly or indirectly be determined by nutritional condition remains unclear. This is important not only for our understanding of how honesty of carotenoid-based sexual signals works but also our understanding of the bewildering variety (see above) of documented effects, or absence thereof, of dietary carotenoid levels.

In a two-way experiment using molting male great tits, *Parus major*, we examined the independent and interactive effects of nutritional state/body condition and dietary carotenoid content. We manipulated dietary quality by providing two types of semi-synthetic food, control and enhanced. Within these diets, we supplemented lutein, the commonest circulating carotenoid in birds and in the great tit (Isaksson et al. 2008), at two (biologically relevant) levels. We measured individual food and lutein consumption, body condition, and circulating carotenoid levels as well as ability to respond to a humoral and inflammatory immune challenge.

Materials and methods

Experimental birds and maintenance

To standardize between experimental subjects, we included only juvenile (recently fledged) males. Between 9 and 27

June 2008, juvenile great tits were caught in mist nets around Möggingen (Radolfzell, Germany; 47°44'N, 08°58'E). Birds were banded with individual aluminium rings, and at this and each subsequent capture, body mass was measured and a blood sample was taken. We also estimated furcular fat scores, but these were too invariant for meaningful analysis (data not shown). The first blood sample was also used for molecular genetic determination of sex (Griffiths et al. 1998). After 1 day in small cloth cages, female juveniles were released and males were moved to individual outdoor aviaries (300 × 300 × 190 cm) with some shrubs, perches and one nest box. During acclimatization to captivity, birds were fed with mealworms plus a mixture of bird seed for wild birds, sunflower seeds, with added vitamin and mineral mixes (Korvimin (WDT) and Vitakalk (Marienfelde)). From 8 to 24 July 2008, these were gradually replaced by a semi-synthetic diet (see below). All birds received food and water ad libitum throughout the experiment. All birds received a treatment against potential infection with coccidians (Kokzidiol, Agraria-Pharma) from 25 July to 7 August 2008.

Blood samples were taken for analysis of plasma carotenoids and hematocrit immediately after capture, during early molt (6 and 7 August 2008) and twice for the SRBC immune test (see later). Blood was collected in heparinized capillary tubes and immediately stored vertically on ice until centrifugation at 13,000g for 5 min in a hematocrit centrifuge (Hettich Hematokrit 210) to determine hematocrit. Plasma was harvested and frozen at −80°C.

Experimental design and diets

In a two-way full-factorial design, we manipulated dietary quality and carotenoid access. Therefore, we used specially formulated semi-synthetic diets of standard (control) and enhanced quality, that contained either low or high concentration of carotenoids (lutein).

The control diet was formulated to simulate a generalist diet with a high content of carbohydrates and fats and adequate protein (Table 1). The enhanced diet was formulated to simulate an optimal insectivore diet that is more nutritious (higher protein, vitamin content) and more easily digestible (lower fibre content, more water) than the control diet. In a 2 × 2 design, both diets were provided with control (low) and high level of added lutein. Due to the higher water content, the birds consumed more of the enhanced diet (Kurvers et al. 2008; Peters et al. 2011; see also “Results”), and therefore we adjusted the lutein content so that the enhanced diet contained 0.10 and 1.00% of lutein powder compared to 0.14 and 1.42% for the control diet (see also Table 1). We assume this to be the only relevant source of lutein in the diet. Although we cannot exclude that the (dried) mealworms may have contained some

Table 1 Composition (% wet weight) of the semi-synthetic diets used in the study

Ingredient	Control carot-low	Control carot-high	Enhanced carot-low	Enhanced carot-high
Water	61.45	60.66	80.73	80.00
Agar	1.23	1.21	2.02	2.00
Glucose	15.12	14.92	3.03	3.00
Casein	7.37	7.28	6.60	6.54
Cellulose	1.92	1.89	–	–
Mineral mix ^a	1.84	1.82	1.18	1.17
Olive oil	7.01	6.92	2.88	2.85
Soy oil	0.37	0.36	0.15	0.15
AA mix ^b	1.03	1.02	0.56	0.55
Vitamin mix ^c	0.15	0.15	0.34	0.33
Mealworms ^d	2.38	2.35	2.42	2.40
Lutein ^e	0.14	1.42	0.10	1.00

Control/enhanced refers to standard and improved diet type, respectively
carot-low low level of added lutein, *carot-high* high added lutein level

^a Custom mineral mix, Dyets Inc

^b Amino acid mix (following the recipe of Murphy and King 1982)

^c AIN vitamin mixture 76, MP Biomedicals Inc

^d Freezedried, Futtermittel Hungenberg

^e FloraGLO, Pfannenschmidt, lutein content: 5%

carotenoids, depending on what they had been fed with, generally mealworms contain only traces of carotenoids (Isaksson and Andersson 2008).

Birds ($n = 56$) were randomly allocated to four different dietary treatments. Before the beginning of the experiment, birds allocated to the different treatment groups did not differ in body mass, hematocrit and plasma carotenoid level (all $P \geq 0.38$). In the course of the experiment, sample size was reduced to 41 due to some birds escaping ($n = 10$) or dying (of unknown causes as established by autopsy, $n = 5$).

Although we provided the experimental diet treatment during the first moult into adult plumage, most males had initiated the moult of the yellow breast before the transition to the semi-synthetic diet. Consequently, we were unable to determine the effects of the diets on yellow plumage coloration, beyond noting that the color appeared within the normal range [mean carotenoid chroma, an index of plumage yellowness, 0.50 ± 0.02 , which is similar to wild great tits captured in the local area (0.54 ± 0.02 , unpublished data from Delhey et al. 2010a); see also Isaksson and Andersson 2008]. Studies on great tits across Europe (nestlings and adults), however, have universally shown that lutein supplementation results in yellower, more chromatic ventral plumage (Eeva et al. 2008, 2009; Fitze et al. 2003, 2007; Isaksson and Andersson 2008; Isaksson et al. 2006; Tschirren et al. 2003; see also Delhey et al. 2010b for effects of a similar diet in blue tits, *Cyanistes caeruleus*).

Food consumption rate

Individual food consumption rate was determined on 13 days (15, 16, 17 and 18 August; 3, 4, 5, 16, 17, 18 and 30 September; 1 and 2 October) before immunization, and on 3 days (13, 14 and 15 October) during SRBC immunization. On these days, all food dishes were weighed before providing them to the birds and again after 24 h. Consumed food was calculated relative to an evaporation control (average evaporation was calculated from three identical control dishes for each diet type placed in unused aviaries). Individual lutein consumption rates were calculated for each diet type separately based on exact lutein content.

Immune responsiveness

To test humoral (antibody) responsiveness, birds were immunized with sheep red blood cells (SRBC), an innocuous complex antigen that generates a robust T-cell-dependent humoral immune response that has been frequently used in immuno-ecological studies (for review, see Snoeijs et al. 2007). We intra-peritoneally injected 50 μ l of a 5% SRBC solution on 9 October 2008 after a pre-immunization blood sample was taken. One week later, on 16 October, a second blood sample was taken and used for the determination of the antibody titer in a haemagglutination assay (Hudson and Hay 1976); 12 of 41 birds responded by producing antibodies (titers: median = 2, range 1–10). Such a relatively low proportion of responders is usual for

the primary immunization with SRBC in wild-caught birds (e.g., Aguilera and Amat 2007; Catoni et al. 2008b; Hanssen et al. 2004; Peters 2000; Roberts and Peters 2009).

To estimate the local inflammatory immune response, on 23 October 2008, we injected 50 μ l of a suspension 0.5 mg/ml PHA-P (Sigma) in the left wing web after its thickness had been measured by using a pressure-sensitive spessimeter. The wing web swelling in response to the PHA injection was measured in the same way 24 h later and the response expressed as the difference between these two values. This immune challenge involves both the adaptive and innate components of the immune system, and it the most widely used immune test in immuno-ecology (for review, see Martin et al. 2006).

Carotenoid concentration

We analyzed the carotenoid (lutein) content of all plasma samples by adding 15 μ l of plasma to 150 μ l of ethanol (absolute, analytical grade), centrifuging at 3,500g for 5 min, and determining absorbance of 100 μ l supernatant at 450 nm in a plate reader (VersaMax, Molecular Devices). We calculated lutein concentration from the absorption coefficient of lutein in ethanol (0.255 ml/ μ g/cm). Concentrations ranged from 18.0 to 44.7 μ g/ml.

Statistical analyses

We analyzed experimental effects in full-factorial GLMs that we reduced by stepwise exclusion of non-significant terms. If we identified significant effects of diet quality or lutein content, when possible we explored these further by examining corresponding effects of individual variation in food or lutein consumption, body mass or plasma lutein concentration. Humoral antibody responsiveness was coded as a binary variable (yes/no) and analyzed using logistic regression models. Maintenance of body condition and plasma carotenoid levels was analyzed as body mass post-immunization–body mass pre-immunization and plasma lutein post-immunization–lutein pre-immunization, respectively; two outliers had to be excluded that showed a change in body mass >2 SD from the mean and that strongly distorted the residuals of the regression models—not excluding these did not qualitatively affect the results. Since there was very high individual consistency over the experiment in individual food (repeatability = 0.69, $F_{43,522} = 29.9$, $P < 0.0001$) and carotenoid (repeatability = 0.86, $F_{43,522} = 82.1$, $P < 0.0001$) consumption rate, to compare food and lutein consumption rates between diet groups, we used an average value from the 13 estimates available for this period (15.08–02.10).

Results

Food consumption rates were higher when birds were provided with the enhanced diet (control diet: 11.2 ± 0.5 g/bird/day; enhanced diet 26.1 ± 1.5 g/bird/day ($F_{1,41} = 88.3$, $P < 0.0001$). This did not vary with lutein level ($F_{1,41} = 0.63$, $P = 0.54$; interaction $F_{1,40} = 1.29$, $P = 0.26$). As a result, lutein consumption rate varied significantly between the four dietary categories (interaction between diet quality and lutein level: $F_{1,40} = 3.30$, $P = 0.002$; Fig. 1a): carotenoid consumption was highest in the enhanced diet with high lutein level (12.2 ± 0.5 mg/day), intermediate in the control diet with high lutein level (8.2 ± 0.5 mg/day) and the enhanced diet with low lutein level (1.4 ± 0.5 mg/day) and lowest in the control diet with low lutein level (0.76 ± 0.48 mg/day).

Birds that were fed the enhanced diet (18.0 ± 0.2 g, $n = 22$) were significantly heavier ($F_{1,40} = 9.86$, $P = 0.003$) compared to males receiving the control diet (17.2 ± 0.2 g, $n = 20$) irrespective of carotenoid content of the diet (carotenoid level: $F_{1,39} = 0.33$, $P = 0.57$; interaction $F_{1,38} = 0.29$, $P = 0.60$). Additionally, they tended to have higher hematocrit (enhanced: 47.4 ± 0.6 , $n = 20$; control: 45.7 ± 0.7 , $n = 20$; $F_{1,38} = 3.52$, $P = 0.07$) irrespective of carotenoid content of the diet (lutein level: $F_{1,37} = 2.31$, $P = 0.14$; interaction: $F_{1,36} = 0.04$, $P = 0.85$).

Plasma lutein concentration (before SRBC immunization) varied significantly with general diet quality and carotenoid level (Fig. 1b): it was increased not only by high lutein content ($F_{1,36} = 11.1$, $P = 0.002$, $\beta = 2.51 \pm 0.75$) but even more so by enhanced quality of the diet ($F_{1,36} = 32.9$, $P < 0.0001$, $\beta = 4.25 \pm 0.74$) with a non-significant trend for an interaction between lutein content and diet quality ($F_{1,35} = 3.13$, $P = 0.09$).

Not unexpectedly, greater individual lutein consumption resulted in higher circulating plasma lutein [individual average lutein consumption in the days (30.09–02.10) preceding plasma sampling: $F_{1,36} = 10.6$, $P = 0.003$]. In addition, there was a very strong positive effect of diet quality ($F_{1,36} = 29.3$, $P < 0.0001$) on plasma carotenoid levels. This result was similar when using average lutein consumption over the past 2 months (diet: $F_{1,36} = 20.2$, $P < 0.0001$; average daily lutein consumption: $F_{1,36} = 9.2$, $P = 0.004$).

Immunity

Likelihood of producing an antibody response following SRBC immunization, was not affected by general diet quality ($\chi^2 = 2.2$, $P = 0.14$) or lutein level ($\chi^2 = 0.26$, $P = 0.61$) nor was antibody titre of responders (diet quality: $\chi^2 = 0.13$, $P = 0.72$; lutein level: $\chi^2 = 2.5$, $P = 0.11$). However, there was a significant difference in condition maintenance (see

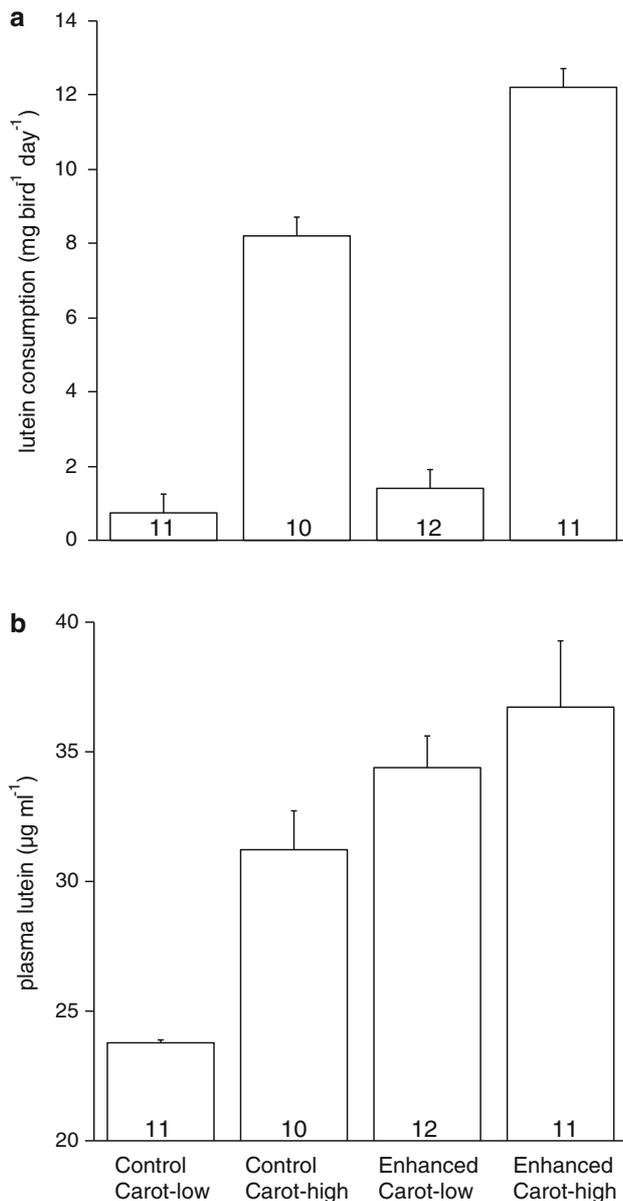


Fig. 1 Plasma lutein levels are increased in great tits, *Parus major*, by improved diet quality (enhanced vs. control) as well as increased general lutein content of the diet (high vs. low) but do not fully match the pattern of lutein consumption rates. **a** Mean \pm SE lutein consumption rates (mg/bird/day) for the four diet groups; **b** corresponding circulating lutein levels (mean \pm SE, $\mu\text{g/ml}$). For lutein consumption, the interaction between diet quality and lutein content was significant ($P = 0.002$), whereas for circulating lutein levels it was not ($P = 0.09$), with highly significant effects of general diet quality ($P < 0.0001$) and lutein content of the diet ($P = 0.002$). *Control* and *enhanced* refers to standard and improved diet type, respectively; *Carot-low* low level of added lutein; *Carot-high* high added lutein level. See text for further details

Fig. 2a) depending on lutein level of the diet ($F_{1,37} = 7.24$, $P = 0.01$): birds that received high levels of lutein gained mass during the week after immunization (0.27 ± 0.08 g) while birds receiving low levels of lutein in their diet did

not (0.02 ± 0.06 g). Maintenance of condition did not vary with diet quality type ($F_{1,36} = 0.01$, $P = 0.93$; interaction: $F_{1,35} = 2.38$, $P = 0.13$). Pre-immunization individual plasma lutein level did not predict individual body mass maintenance ($F_{1,36} = 1.75$, $P = 0.20$). After immunization, birds maintained constant plasma carotenoid levels (change: -0.37 ± 0.83 , $t_{37} = -0.44$, $P = 0.66$) and the individual change in plasma lutein levels was not affected by lutein supplementation ($F_{1,29} = 0.45$, $P = 0.51$), diet type ($F_{1,29} = 0.31$, $P = 0.58$) or their interaction ($F_{1,27} = 0.15$, $P = 0.70$).

The swelling in response to PHA injection (mean 0.44 ± 0.03 , range 0.13–1.1 mm; see Fig. 2b) was higher in birds that received a diet with high levels of carotenoids (0.53 ± 0.05 mm) compared to males that received only modest amounts of lutein in the diet (0.38 ± 0.04 mm, $F_{1,39} = 5.79$, $P = 0.02$). Swelling did not vary with general diet quality ($F_{1,38} = 0.23$, $P = 0.63$; interaction: $F_{1,37} = 0.04$, $P = 0.85$). Individual carotenoid concentration (measured before and after the SRBC immunization) did not predict the inflammatory response (plasma lutein level on 16 October: $F_{1,38} = 0.81$, $P = 0.42$; 9 October: $F_{1,38} = 0.22$, $P = 0.83$).

Discussion

The experimental diets manipulated condition and carotenoid consumption rate as intended. Condition (body mass, hematocrit) was elevated in the birds receiving the enhanced diet, and daily carotenoid consumption was increased in birds receiving the higher dose of lutein. Plasma lutein levels were within the natural range of those observed in free-living male great tits at this time of year (Isaksson et al. 2007; Fig. 1, post-breeding males). The positive effect of carotenoid supplementation on circulating levels in plasma was not surprising, and has been demonstrated in all investigated avian species (e.g., Alonso-Alvarez et al. 2004; Hörak et al. 2006; McGraw et al. 2006; and references therein), including great tits (Isaksson and Andersson 2008). Many (but see Alonso-Alvarez et al. 2004) of these studies presented the rather artificial situation of diets with and without access to carotenoids, and it is reassuring that providing diets with different, naturalistic levels of carotenoid content likewise affected circulating carotenoids.

Plasma lutein concentration was not only determined by lutein level of the diet, but even more so by general diet quality (enhanced vs. control; see Fig. 1b). A greater effect of diet quality on plasma carotenoid concentration than the effect of ingested lutein was unexpected. Although plasma levels of carotenoids are not only affected by uptake but also by mobilization of carotenoid stores, for example from the liver, this effect is likely to be much smaller than effects

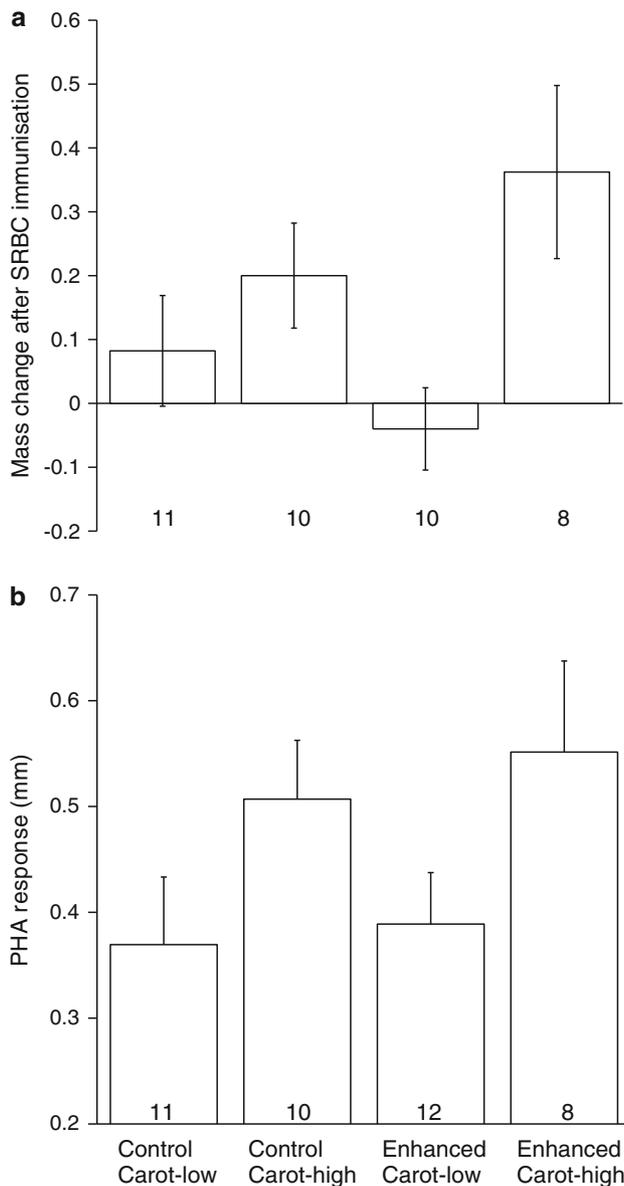


Fig. 2 Health benefits of carotenoid consumption. Dietary lutein supplementation alleviates the somatic cost of a humoral immune challenge and improves inflammatory immune response. **a** Maintenance of body mass during SRBC immunization [mean \pm SE change in body mass (g) during the week following injection with SRBC]; **b** size of the inflammatory response to PHA injection [mean swelling (mm) \pm SE]. Effects of lutein levels, but not general diet quality, were statistically significant (both $P \leq 0.02$). *Control* and *enhanced* refers to standard and improved diet type, respectively; *Carot-low* low level of added lutein; *Carot-high* high added lutein level. See text for details

of dietary carotenoid levels (Isaksson and Andersson 2008). Therefore, our results indicate that the ability of male great tits to absorb lutein from the diet into plasma circulation was improved by an enhanced diet. An effect of food intake on carotenoid absorption was previously demonstrated in American goldfinches, *Carduelis tristis*. When

male goldfinches were subject to a nutritional stress regime (temporary removal of all available food), this resulted in a reduction in circulating carotenoid levels, despite similar carotenoid intake (McGraw et al. 2005). Although it seems likely that this result was directly linked to nutritional state, it cannot be excluded that food deprivation is seen as a stressor (Lynn et al. 2003), and that stress might reduce carotenoid uptake from food or increase carotenoid allocation to storage organs (Eraud et al. 2007).

Our results indicate that the ability of male great tits to absorb lutein from the diet into plasma circulation is highly sensitive to nutritional state and/or diet composition. This effect could be related to qualities of the food ingested with the lutein, either to better general digestibility of the enhanced diet or to specific components. Fat content of the diet seems at first sight a likely candidate, since carotenoids are fat soluble and transported by blood lipoproteins (based on cholesterol), and cholesterol content of the diet can affect plasma carotenoid levels (McGraw and Parker 2006) and vice versa (Hörak et al. 2006). However, although fat content of the control diet was 2.5 times higher, since birds consumed almost 2.5 times as much of the enhanced diet, daily fat consumption for both diet types was nearly identical (fat sources were the same). Another candidate dietary component for explaining our results is protein, an important determinant of condition. However, in a 2×2 manipulation of dietary protein and carotenoid content of pheasants, *Phasianus colchicus*, Smith et al. (2007) did not find that the positive effect of carotenoids on wattle color or immunity was improved by elevated dietary protein levels, but they did not investigate plasma carotenoid levels. Finally, the great tits provided with the enhanced diet consumed on average 5.2 times more of the vitamin mix (Table 1). Since carotenoid and vitamins (mostly E and C) can interact competitively during absorption and positively post-absorption (Catoni et al. 2008a), vitamin content of the diet might be (partly) responsible for the observed dietary effect on plasma carotenoid levels. Only further, more precise, manipulations could test the suggestion that specific composition of the food with which carotenoids are ingested might affect their uptake.

Additionally or alternatively, the observed effect of general diet quality on plasma carotenoids might be related to improved general condition of the birds that received the enhanced diet. Since carotenoid-based coloration depends on feather carotenoid content (Saks et al. 2003) and since carotenoid deposition in the feather is determined by circulating plasma levels (McGraw 2006), our results are in agreement with studies showing that carotenoid-based coloration is improved with increased individual condition (Hill and Montgomerie 1994), and that this can be partly independent of carotenoid access (Hill 2000). Notably, Smith et al. (2007) showed that male pheasants showed a

positive correlation between wattle color and body condition, but only when provided with high levels of dietary carotenoids. This indicates that pheasants in better condition could assimilate more carotenoids, or that birds that accumulated more carotenoids were in better condition. Likewise, Tschirren et al. (2003) showed in an experimental supplementation experiment with great tit nestlings that the ability to incorporate carotenoids into the yellow feathers depends, besides the access to carotenoids, on general condition. They hypothesized that this result indicates that incorporation of carotenoids in feathers is costly. Our finding that circulating carotenoid levels are higher in birds in better condition could provide an alternative, testable mechanistic explanation for their observation.

Immunity

Higher lutein intake, but not greater general diet quality, positively affected how well birds coped with a humoral immune challenge: birds that had consumed food with a higher lutein content during the preceding months were able to increase their body mass during the period of antibody production. Loss of body mass/condition is an important cost of humoral immune activation that can have far-reaching fitness consequences (Eraud et al. 2005; Hanssen 2006; Hõrak et al. 2006). Like in our great tits, carotenoid supplementation of greenfinches, *Carduelis chloris*, could alleviate this somatic cost of immune activation, i.e. immune challenge reduced body-mass gain, but only among the carotenoid-depleted birds (Hõrak et al. 2006). Some studies report that carotenoid supplementation results in an increase in antibody production in response to SRBC immunization (Hõrak et al. 2006; McGraw and Ardia 2003, 2005; but see Navara and Hill 2003) or that antibody production results in a decline in circulating carotenoid levels (Aguilera and Amat 2007; Alonso-Alvarez et al. 2004; Peters et al. 2004; but see Hõrak et al. 2006). Most studies do not include all three possible interactions between immunization and carotenoid supplementation in their analyses (but see Hõrak et al. 2006 for an exception), so we cannot assess which effects are more likely to occur, and which aspect of self-maintenance birds are likely to favour under what conditions. However, it is becoming increasingly clear that increased carotenoid consumption has a beneficial effect during a humoral immune challenge.

Additionally, lutein-supplemented male great tits produced a greater inflammatory response to injection with PHA. Response to PHA has been widely used in ecological studies, and it has been shown to vary with individual quality and condition, hormone levels, and parasitism as well as a variety of environmental conditions, pollution, and food availability or quality (for review, see Martin et al. 2006). We did not find a general effect of diet quality or condition,

and possibly such links are more likely to be detected in free-living birds. However, we did find an expected positive effect of increased lutein consumption. A positive effect of carotenoid supplementation on the wing web swelling index that has been found in a variety of avian species, particularly male zebra finches, *Taeniopygia guttata* (Blount et al. 2003; McGraw and Ardia 2003, 2005) but also growing chickens (Koutsos et al. 2007) and great tit nestlings (Fitze et al. 2007). On the other hand, no such effect was found in related finches lacking carotenoid-based ornamentation (McGraw et al. 2006), laying chickens (Bedecarrats and Leeson 2006) or male blue tits (receiving a very similar diet as in the birds in the present study; Roberts and Peters 2009). The causes of these differences for the moment remain obscure, until more supplementation studies have been performed.

Individual plasma levels of carotenoids did not predict the observed treatment effects on immunity: there was no effect of pre-immunization plasma lutein level on PHA-induced wing web swelling (whereby plasma lutein was measured 1 week before PHA injection). The few previous studies who have similarly investigated this relationship showed very mixed results: a positive relationship (Perez-Rodriguez et al. 2008), no relationship (Hõrak et al. 2006) and even a negative relationship (Biard et al. 2009). Until more such studies are performed, it is not possible to distinguish whether these differences are species-specific, or related to differences in (carotenoid availability in) the diet or whether general dietary lutein content affects immune allocation, rather than current carotenoid levels in circulation.

Conclusion

There is a large degree of variability in effects of carotenoids, and lively discussion on whether and how carotenoid levels in diet are important for animal health (for some recent reviews, see Alonso-Alvarez et al. 2008; Catoni et al. 2008a; Perez-Rodriguez 2009; Peters 2007). Our results indicate that some of the variation in the results of carotenoid supplementation studies could possibly be related to background diet. The addition of carotenoids integrated into the basic food supply is a much more realistic way of supplementing carotenoids than in the drinking water, as has typically been done in pioneering supplementation studies (for example Blount et al. 2003; McGraw and Ardia, 2003, 2005; Hõrak et al. 2006; Alonso-Alvarez et al. 2004; but see also Isaksson and Andersson 2008; McGraw et al. 2006). Our results indicate that, if effects of carotenoids are dose-dependent on plasma levels (Alonso-Alvarez et al. 2004), some differences between studies might be explained by differences in method of supplementation, or

quality of background diet, particularly when circulating levels of carotenoids have not been measured. However, our results indicate that, even though carotenoids in circulation can be dramatically increased by enhanced diet quality and general condition, the positive effects of carotenoids were dependent on dietary levels, suggesting that allocation of carotenoids to self-maintenance is possibly governed by allocation rules based on dietary carotenoid availability. This could explain some of the contradictory results in the relationship between carotenoid concentration in plasma and experimental results. Above all, it highlights that where carotenoids are concerned, many more experimental and observational studies are needed.

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