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Effects of carotenoids, immune activation and immune suppression on the intensity of chronic coccidiosis in greenfinches

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ABSTRACT

Allocation trade-offs of carotenoids between their use in the immune system and production of integumentary colouration have been suggested as a proximate mechanism maintaining honesty of signal traits. We tested how dietary carotenoid supplementation, immune activation and immune suppression affect intensity of coccidian infection in captive greenfinches *Carduelis chloris*, a passerine with carotenoid-based plumage. Immune activation with phytohaemagglutinin (PHA) decreased body mass among birds not supplemented with lutein, while among the carotenoid-fed birds, PHA had no effect on mass dynamics. Immune suppression with dexamethasone (DEX) induced loss of body mass and reduced the swelling response to PHA. DEX and PHA increased the concentration of circulating heterophils. Lutein supplementation increased plasma carotenoid levels but had no effect on the swelling response induced by PHA. PHA and DEX treatments did not affect plasma carotenoids. Immune stimulation by PHA suppressed the infection, but only among carotenoid-supplemented birds. Priming of the immune system can thus aid in suppressing chronic infection but only when sufficient amount of carotenoids is available. Our experiment shows the importance of carotenoids in immune response, but also the complicated nature of this impact, which could be the reason for inconsistent results in studies investigating the immunomodulatory effects of carotenoids. The findings about involvement of carotenoids in modulation of an immune response against coccidiosis suggest that carotenoid-based ornaments may honestly signal individuals' ability to manage chronic infections.

1. Introduction

Immune system protects an organism against infections by detection and destruction of parasites and pathogens. These processes that involve construction of sophisticated recognition and memory systems, release of harmful substances and increased metabolism, interact and compete with all other functions of an organism. Immune defences thus have their costs, which are currently believed to play important roles in mediating trade-offs associated with life-history and signal traits (Sheldon and Verhulst, 1996; Lee, 2006; Hasselquist, 2007; Schulenburg et al., 2009). This nexus has given a birth to an expanding new discipline – immunoecology.

One of the most intriguing issues in immunoecology concerns the association between carotenoids and immune function. Carotenoids, which are produced mainly by plants, are common integumentary pigments of birds, fishes and reptiles. Although animals cannot synthesise carotenoids *de novo*, carotenoids are extraordinarily common components of the colour of signals used in sexual communication, signalling between offspring and their parents and in warning colours (Møller et al., 2000; Hõrak and Saks, 2003). Examples range from the bright yellow, orange, and red feathers, beaks, legs and gapes to the elaborate colouration of the skin and various body parts. The link between carotenoid-based ornamentation and immune function was first suggested by Lozano (1994) and later developed by von Schantz et al. (1999) who emphasised the antioxidant function of carotenoids and highlighted the connection between ornaments, oxidative stress and immune function. Under this view, 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 maintenance purpose at the same time. Many correlative studies have reported positive associations between the extent of carotenoid ornament expression and different aspects of the activation of immune system (reviewed by Møller et al., 2000; Blount, 2004; McGraw, 2006). Links between immune function and carotenoids have been experimentally demonstrated in at least 12 bird species, either by the positive effect of carotenoid supplementation or by stimulation of immune system that depleted carotenoid stores (reviewed by McGraw et al., 2006; Aguilera and Amat, 2007; Fitze et al., 2007; Perez-Rodriguez et al., 2008;

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Stirnemann et al., 2009). However, results demonstrating the lack of associations between carotenoids and immunity have also started to accumulate (reviewed by Hõrak et al., 2007; Smith et al., 2007).

Studies demonstrating the positive effect of carotenoids on the magnitude of immune responses to artificial antigens are difficult to interpret in ecological context in two reasons. First, maximum immune responses are not necessarily optimal or best for an organism as a whole, because immune response comes with a cost (e.g., Viney et al., 2005). Second, different compartments of the immune system are in cross-regulatory balance, so that enhancement of one arm of the immune system (e.g., humoral) may down-regulate another arm (e.g., cellular) and vice versa (e.g., Graham et al., 2005). The implicit assumption of standard immune challenge protocols that immunoresponsiveness to non-parasitic antigens reflects the ability to resist disease is thus weakly grounded (Owens and Wilson, 1999; Adamo, 2004; Saks et al., 2006). Understanding the potential health benefits of carotenoids therefore requires assessment of the performance of immune system in a functional level, that is, by its ability to manage real infections. However, such an approach has been exceptional as only four studies, listed below, have tested the effect of carotenoids on the development of infections in birds.

In birds, one of the most easily traceable natural infection is intestinal coccidiosis. Coccidians are directly transmitted protozoan parasites that cause production loss in poultry industry (e.g., Zhu et al., 2000) and affect fitness of the wild birds (see Hõrak et al., 2004; Gill and Paperna, 2008). Inhabiting intestinal epithelium, they directly inhibit the uptake of essential dietary components, including carotenoids, and consequently depress carotenoid-based pigmentation (reviewed by Pap et al., 2009). Avian coccidiosis model has been applied for investigation of potential immunomodulatory effects of carotenoids in three studies. Hõrak et al. (2006b) found no effect of dietary lutein supplementation on the dynamics of naturally acquired Isosporan infection in greenfinches. In blackbirds (*Turdus merula*), carotenoid supplementation delayed the relapse of experimental infection by 1 week as compared to the un-supplemented control group, although the infection intensity was unaffected (Baeta et al., 2008). In house sparrows (*Passer domesticus*), carotenoid supplementation had no effect on infection intensity but it significantly reduced the mass loss caused by coccidiosis (Pap et al., 2009). Yet another study on American goldfinches (*Carduelis tristis*) found no effect of carotenoid supplementation on the infection of bacterial pathogen *Mycoplasma gallinarum* (Navara and Hill, 2003). Inconsistency of these findings clearly points for the need for further studies testing the effects of carotenoids to natural infections. Yet unexplored possibility to examine the role of carotenoids in immune response is through controlled immune suppression and immune activation experiments. For instance, it may appear possible that the impact of carotenoids on natural infection levels is detectable only during immune suppression caused by stress or after previous activation of immune system.

Here we examine the effect of dietary carotenoid supplementation on the dynamics of natural Isosporan infection on greenfinches. Greenfinches are about 30 g sexually dichromatic seed-eating passerines that display a variety of carotenoid-based plumage patches, ranging from bright yellow to olive. The colour of contrasting yellow tail patch is sensitive to infections, reflects immune responsiveness to foreign antigens and is strongly associated with plasma lutein concentration and general physiological condition (reviewed by Aguilera and Amat, 2007; Peters et al., 2008). In order to amplify the potential effects of carotenoid supplementation, we performed immune activation and immune suppression treatments, injecting supplemented and un-supplemented birds with either phytohaemagglutinin (PHA) or dexamethasone (DEX) in a factorial design. Specifically, we assumed that possible immunostimulatory effects of carotenoids are more likely to emerge when immune system is suppressed by a synthetic corticosteroid DEX. Acting on glucocorticoid receptors, DEX mimics the effects of corticosterone as a part of the feedback mechanism that turns immune activity (inflammation) down (see Holberton et al., 2007). In birds, DEX-induced immune suppression has been previously used to study the effects of infection (e.g., Huff et al., 1999) or validation of immunotoxicological techniques (Smits and Williams, 1999) or manipulation of corticosterone levels (e.g., Rich and Romero, 2005), however, to our knowledge DEX has been never used to study the immunomodulatory effects of carotenoids. Because the physiological (Alonso-Alvarez et al., 2004) and immunomodulatory (Krinsky and Johnson, 2005) effects of carotenoids can be dose-dependent, we applied dietary carotenoid supplements differing in the concentration of lutein by two times (i.e., 8 and 16 μg/ml in addition to lutein-free control treatment). PHA activates the same cytokines that are involved in immune response against coccidiosis (Lillehoj, 1998). We therefore asked whether the priming of the immune system with PHA induces stronger immune response against coccidiosis which helps to suppress the infection, and how this possible interaction is affected by carotenoid supplementation. PHA treatment also enabled performing replicate test of previous studies, showing that carotenoid supplementation increased an immune response to PHA. In order to detect the effects of treatments upon immune system and general condition of birds, we measured the changes in coccidian infection intensity, swelling response to PHA and dynamics of body mass and peripheral immune cell counts.

2. Methods

Male greenfinches ($N = 93$) were caught in mist-nets in the Sõrve Bird Observatory in Estonian island Saaremaa (57° 55′ N; 22° 03′ E) on 25–27 January, 2007. Birds were transported to Tartu and housed indoors in individual cages (27 × 51 × 55 cm) with sand bedding. Average temperature in the aviary during the experiment was 15.9 ± 1.7 (SD) °C and average humidity was 53.2 ± 2.6 (SD) %. The birds were supplied *ad libitum* with sunflower seeds and filtered tap water. Birds were held on the natural day-length cycle on artificial lighting. Blood samples were collected with a six-day interval on 21 and 27 February (Fig. 1) in the morning before the lights turned on. Captive birds were released into their natural habitat in 1 April. The study was conducted under the license from the Estonian Ministry of the Environment and the experiments comply with the current laws of Estonian Republic.

On 6 February, birds were divided to three treatment groups which were set to have similar average body mass at capture and age composition. Thirty three birds started to receive high dose carotenoid supplementation, 32 birds received low dose of carotenoids, and 28 birds (controls) received filtered tap water. Supplementation consisted of 18 (high dose) or 6 (low dose) μg/ml water solution of lutein and zeaxanthin (20:1, w/w), prepared from OroGlo liquid solution of 11 g/kg xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium). Those solutions were freshly prepared each evening using filtered (Brita® Classic; BRITA

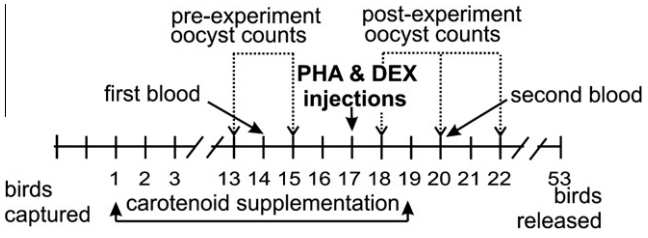

Fig. 1. Course of the experiment. Day 1 = 7 February.

GmbH, Taunusstein, Germany) tap water and were provided in 30 mL doses in opaque dispensers in order to avoid oxidation of carotenoids. Carotenoid supplementation lasted 19 days, covering blood sampling events.

In the evening of 24 February (on the fourth day after first blood sampling and 3 days before second blood sampling), birds in all three carotenoid treatment groups were assigned to 2×2 treatments of immune challenge and immune suppression. Half of birds from each treatment group were injected intradermally in the wing web with 0.2 mg of phytohaemagglutinin (PHA; Sigma, St. Louis, MO, L-8754) in 0.04 mL of sterile isotonic saline. At the same time, the rest of the birds were injected with saline. These treatment groups were again split by half, so that half of the group received an injection of immunosuppressant DEX. We thus ended up with $2 \times 2 \times 3$ design of immune activation, immune suppression and carotenoid supplementation (three levels) with 7–8 birds per treatment category. DEX (KRKA, d.d., Novo Mesto, Slovenia; 0.03 mg in 0.05 mL sterile isotonic saline; set to approximate 1 mg/kg body weight) was injected into pectoralis muscle while the rest of the birds received the same amount of isotonic saline injection. DEX injection took place immediately after PHA or saline injection into wing web.

Birds were sampled for coccidian (*Isospora* sp.) infection by faecal examination on 20 and 22 February (pre-experiment oocyst counts) and on 25 and 27 February and 1 March (post-experiment oocyst counts). Infection intensities were averaged over the pre- and post-experimental period. All birds appeared naturally infected. Infection intensities (number of oocysts per gram faeces) of individual greenfinches were quantified as described in Hõrak et al. (2004) and Hõrak et al. (2006a).

For counting leukocytes, a drop of blood was smeared on microscope slide, air-dried, fixed in absolute methanol, and stained with azure-eosin (Romanowsky stain). We estimated the proportion of different types of leukocytes by examining 100 leukocytes under 1000× magnification under oil immersion. Estimates of the total white blood cell count (WBC) were obtained by counting the number of leukocytes per approximately 10,000 erythrocytes. Differential leukocyte counts were obtained by multiplying their proportions with WBC. Repeatabilities (Lessells and Boag, 1987) of leukocyte counts, obtained from a sample of smears counted twice were: WBC; $r = 0.82$, $F = 10.0$, df = 19,20, $P < 0.001$; heterophil concentration: $r = 0.79$, $F = 8.4$, df = 19,20, $P < 0.001$; lymphocyte concentration: $r = 0.82$, $F = 10.1$, df = 19,20, $P < 0.001$; heterophil/lymphocyte (H/L) ratio: $r = 0.79$, $F = 8.5$, df = 19,20, $P < 0.001$. For all calculations, leukocyte count values were ln-transformed.

Plasma was separated from the whole blood, samples were stored at −80 °C until analysed. Concentration of carotenoids was determined spectrophotometrically from supernatant obtained from centrifuging 15 μl plasma diluted in 150 μl of acetone as described by Tummeleht et al. (2006).

Effects of experimental treatments upon changes in physiological parameters were assessed in factorial ANCOVAs adjusting for initial trait values. We started examination of full models with interaction between factors, dropping non-significant effects one at a time. In the final models, presented in the right side of Table 1, only significant main effects and interactions were retained. Pairwise differences between treatments were tested in Tukey HSD tests for unequal sample sizes and significant contrasts are indicated on the figures. Assumptions for parametric models (normality of residuals, homogeneity of variances) were met for all measured parameters except plasma carotenoid concentration. However, this variable was affected only by carotenoid supplementation, an effect that was also revealed by a non-parametric test. Sample sizes differ between some analyses due to our inability to record all parameters from all the birds. All tests are two-tailed with a *P*-level below 0.05 as a criterion for significance.

3. Results

DEX-injected birds lost more body mass than the rest of the birds (−2.78 ± 1.51 g, ($n = 44$) vs −2.03 ± 2.32 g ($n = 47$; Table 1)). Effects of PHA injection on body mass interacted with carotenoid supplementation: among un-supplemented birds PHA decreased mass while among the birds supplemented with carotenoids, PHA had no effect on mass dynamics (Fig. 2). Carotenoid supplementation did not affect the swelling response to PHA (Table 1), but DEX treatment significantly reduced swelling (DEX: 0.37 ± 0.24 mm ($n = 22$) vs Non-DEX: 0.54 ± 0.24 mm ($n = 24$). None of the treatments had direct effect on intensity of coccidiosis, however, there was a significant three-way interaction between all treatments (Table 1). The same model hold when we simplified the design by pooling the birds on low and high carotenoid diet into the one group. Fig. 3 indicates that infection decreased most among the birds receiving carotenoids and PHA but no DEX injection.

None of the treatments affected the change in plasma carotenoid levels in ANCOVA (Table 1), but since the carotenoid supplementation had started before the first blood sampling, plasma carotenoid levels in both supplemented groups were higher than in un-supplemented birds (Fig. 4). Comparison of individual carotenoid profiles showed a decline in plasma carotenoids among un-supplemented birds and those supplemented with low carotenoid dose. Dose of carotenoids (high vs low) had no significant effect on plasma carotenoid levels (Fig. 4). PHA injection marginally increased total WBC (Table 1), which was evidently due to increase in heterophil count (Fig. 5). DEX and PHA increased heterophil concentration in an additive manner (Table 1, Fig. 5). None of the treatments affected lymphocyte concentration. DEX treatment significantly increased H/L ratio (DEX: 0.37 ± 0.39 ($n = 26$) vs Non-DEX 0.17 ± 0.31 ($n = 26$)).

4. Discussion

All experimental treatments had clear physiological effects. Dietary supplementation increased plasma carotenoid levels. This effect was, however, independent of dietary carotenoid concentration, which may explain the lack of dose-dependent effect of carotenoids on other studied parameters. DEX suppressed T-cell mediated swelling response to PHA and also reduced body mass and increased peripheral heterophil concentrations. These findings are in accordance with previous studies in birds (Schrank et al., 1990; Fowles et al., 1993; Isobe and Lillehoj, 1993; Huff et al., 1999; Smits and Williams, 1999). Expectedly, PHA injection stimulated immune system as indicated by increased heterophil count. The effect of immune stimulation by PHA on body mass depended on the dietary carotenoids: among un-supplemented birds PHA decreased mass while among the carotenoid-supplemented birds, PHA had no effect on mass. This finding suggests that carotenoid supplementation can modulate the physiological impact of immune activation. If birds are low in carotenoids, the immune challenge implies somatic costs in terms of mass loss. On the other hand, we found no evidence that carotenoid supplementation had generally attenuated mass loss. Decline of body mass due to immune challenge in this study is similar to findings of Hõrak et al. (2006b) showing that injection of sheep red blood cells reduced body mass only among the birds not supplemented with carotenoids. Replication of this result with different kind of immune activator suggests that beneficial effects of carotenoids on maintenance of body mass may appear similar in case of humoral and inflammatory challenges. Reduction in body mass, mass gain or growth in response to non-infectious immune challenge have been documented in several avian studies (reviewed by Hanssen, 2006; Hõrak et al., 2006b). Such effects can be caused either by depletion of bodily resources to support development of energetically demanding inflammatory response or reduction of food intake

Table 1
Effects of carotenoid, PHA and DEX treatments on changes of body mass, plasma carotenoids and immune parameters of the birds between days 15 day 20 of the experiment. Leukocytes were sampled only from the birds with high level of dietary carotenoids and un-supplemented birds.

Variable	Initial model Predictor	F_{df}	p	Final model Predictor	F_{df}	p
Mass	Initial value	$24.1_{1,76}$	<0.0001	Initial value	$24.2_{1,81}$	<0.0001
	DEX	$4.5_{1,76}$	0.037	DEX	$4.4_{1,81}$	0.039
	PHA	$0.05_{1,76}$	0.82	PHA	$0.03_{1,81}$	0.87
	Carot	$2.8_{2,76}$	0.24	Carot	$1.4_{2,81}$	0.25
	DEX × PHA	$0.1_{1,76}$	0.71	Carot × PHA	$4.3_{2,81}$	0.016
	Carot × PHA	$4.0_{2,76}$	0.023	–	–	–
	Carot × DEX	$1.3_{2,76}$	0.28	–	–	–
	Carot × DEX × PHA	$1.3_{2,76}$	0.27	–	–	–
Swelling response to PHA	DEX	$5.3_{1,40}$	0.027	DEX	$5.6_{1,44}$	0.023
	Carot	$1.2_{2,40}$	0.32	–	–	–
	Carot × DEX	$1.52_{2,40}$	0.24	–	–	–
Oocyst counts	Initial value	$64.4_{1,82}$	<0.0001	Initial value	$73.2_{1,85}$	<0.0001
	DEX	$0.04_{1,82}$	0.85	DEX	$0.1_{1,85}$	0.75
	PHA	$0.5_{1,82}$	0.50	Carot	$0.4_{1,85}$	0.52
	Carot	$0.4_{1,82}$	0.53	PHA	$0.4_{1,85}$	0.51
	DEX × PHA	$0.2_{1,82}$	0.64	Carot × DEX × PHA	$5.6_{1,85}$	0.021
	Carot × PHA	$0.06_{1,82}$	0.81	–	–	–
	Carot × DEX	$0.07_{1,82}$	0.80	–	–	–
	Carot × DEX × PHA	$5.4_{1,82}$	0.022	–	–	–
Carotenoids	Initial value	$1.6_{2,71}$	0.21	–	–	–
	DEX	$0.2_{1,71}$	0.64	–	–	–
	PHA	$0.8_{1,71}$	0.38	–	–	–
	Carot	$2.68_{2,71}$	0.075	–	–	–
	DEX × PHA	$1.0_{1,71}$	0.31	–	–	–
	Carot × PHA	$1.7_{2,71}$	0.19	–	–	–
	Carot × DEX	$1.1_{2,71}$	0.34	–	–	–
	Carot × DEX × PHA	$0.6_{2,71}$	0.54	–	–	–
Total WBC	Initial value	$45.3_{1,43}$	<0.0001	Initial value	$44.9_{1,49}$	<0.0001
	DEX	$0.6_{1,43}$	0.43	PHA	$3.9_{1,49}$	0.053
	PHA	$3.1_{1,43}$	0.087	–	–	–
	Carot	$0.4_{1,43}$	0.56	–	–	–
	DEX × PHA	$0.5_{1,43}$	0.46	–	–	
	Carot × PHA	$1.9_{1,43}$	0.17	–	–	–
	Carot × DEX	$1.1_{1,43}$	0.29	–	–	–
	Carot × DEX × PHA	$0.8_{1,43}$	0.38	–	–	–
Heterophils	Initial value	$61.1_{1,43}$	<0.0001	Initial value	$66.2_{1,48}$	<0.0001
	DEX	$6.1_{1,43}$	0.018	DEX	$6.2_{1,48}$	0.017
	PHA	$5.6_{1,43}$	0.023	PHA	$6.1_{1,48}$	0.017
	Carot	$0.1_{1,43}$	0.80	–	–	–
	DEX × PHA	$1.7_{1,43}$	0.20	–	–	–
	Carot × PHA	$0.9_{1,43}$	0.35	–	–	–
	Carot × DEX	$0.4_{1,43}$	0.52	–	–	–
	Carot × DEX × PHA	$0.03_{1,43}$	0.87	–	–	–
Lymphocytes	Initial value	$46.6_{1,43}$	<0.0001	–	–	–
	DEX	$<0.01_{1,43}$	0.99	–	–	–
	PHA	$0.9_{1,43}$	0.35	–	–	–
	Carot	$0.7_{1,43}$	0.39	–	–	–
	DEX × PHA	$0.1_{1,43}$	0.75	–	–	–
	Carot × PHA	$0.8_{1,43}$	0.39	–	–	–
	Carot × DEX	$1.6_{1,43}$	0.21	–	–	–
	Carot × DEX × PHA	$1.1_{1,43}$	0.31	–	–	–
H/L ratio	Initial value	$49.1_{1,43}$	<0.0001	Initial value	$54.3_{1,49}$	<0.0001
	DEX	$7.1_{1,43}$	0.011	DEX	$8.6_{1,49}$	0.005
	PHA	$2.6_{1,43}$	0.111	–	–	–
	Carot	$0.4_{1,43}$	0.56	–	–	–
	DEX × PHA	$1.5_{1,43}$	0.22	–	–	–
	Carot × PHA	$0.23_{1,43}$	0.63	–	–	–
	Carot × DEX	$0.3_{1,43}$	0.56	–	–	–
	Carot × DEX × PHA	$1.1_{1,43}$	0.31	–	–	–

and locomotory activity due to inflammation-induced sickness syndrome (reviewed by Boughton et al., 2007; French et al., 2009).

The main aim of this study was to test for the involvement of carotenoids in modulation of the immune response. Carotenoid-supplemented birds did not develop stronger swelling response to PHA than un-supplemented birds. This result compares favourably with our previous experiments with greenfinches (Hõrak et al., 2006b, 2007) and several other avian studies (Navara and Hill, 2003; McGraw and Ardia, 2005; Bedecarrats and Leeson, 2006; McGraw et al., 2006; McGraw and Klasing, 2006). Yet these results do not refute the immunomodulatory potential of carotenoids in birds because associations between immune function and carotenoids have been experimentally demonstrated in at least 12 bird species (see Section 1). Injections by PHA or DEX

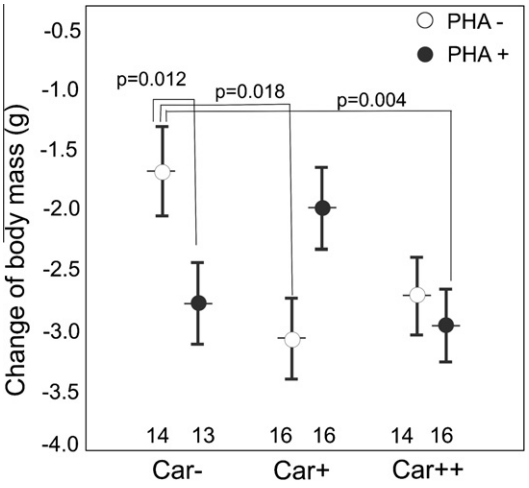

Fig. 2. Effects of carotenoid and PHA treatments on the change of body mass of the birds between day 15 and 20 of the experiment. Least square means ± SE from the final model in Table 1. Unless stated otherwise, significant pairwise differences from Tukey HSD test are always indicated above, sample sizes below whiskers. In all figures Car− stands for un-supplemented birds, Car+ is low dietary carotenoid supplement (6 μg/ml) and Car++ is high dietary carotenoid supplement (18 μg/ml). In all figures, if no *p*-value is shown, then the difference is not statistically significant.

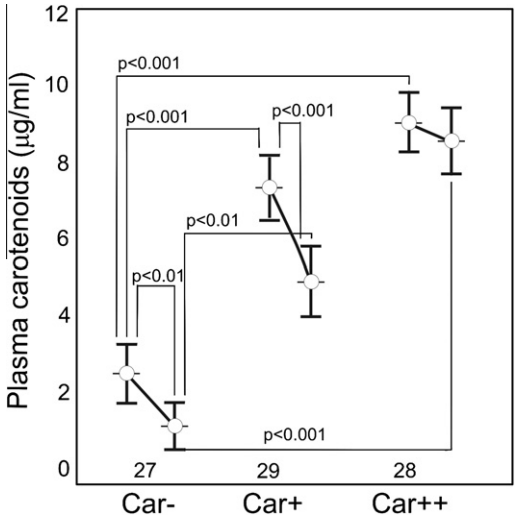

Fig. 4. Effects of carotenoid supplementation on plasma carotenoid levels of birds with respect to dose. Significant individual changes during the experimental period (Wilcoxon tests) are indicated within treatments. *P*-values for between-group differences are from Mann–Witney U-tests.

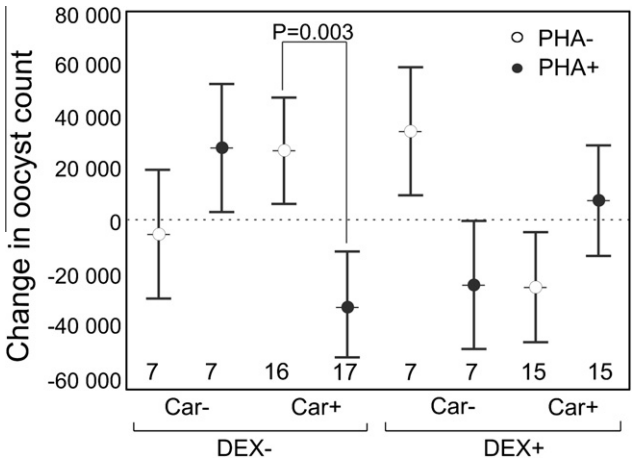

Fig. 3. Effects of carotenoid, PHA and DEX treatments on the change of coccidian infection intensity of the birds between day 15 and 20 of the experiment. Least square means ± SE from the simplified final model where high and low carotenoid treatments were pooled.

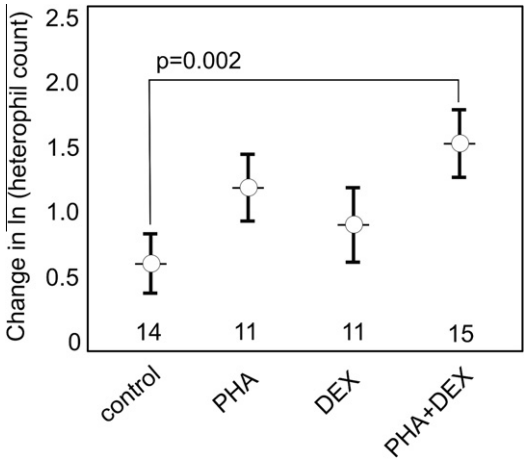

Fig. 5. Effects of carotenoid and PHA treatments on the change of heterophil hemoconcentration during the experiment.

did not deplete plasma carotenoids either. This result again compares favourably with our previous experiments on greenfinches but is opposite to that found in five other avian studies using different immunostimulants (reviewed by Perez-Rodriguez et al., 2008). However, two recent studies have also hinted that the timing of measurement is important in detection of the carotenoid depletion by immune challenge. In red-legged partridges (*Alectoris rufa*) Perez-Rodriguez et al. (2008) found a 13% decline in circulating carotenoids 24 h after injection. Biard et al. (2009) found significant decline in circulating carotenoids in 24 and 48 h after PHA injection, but not on the fourth day post injection (PI). In this study we sampled plasma carotenoids 4 days PI and previously either 3 days PI (Hõrak et al., 2007) or 16 days PI (Hõrak et al., 2006b). It may thus well appear that possible treatment effects on plasma carotenoids remained undetected because blood sampling took place too late after immune challenge.

Treatments with DEX, PHA or carotenoids had no significant main effects on the development of coccidiosis. Birds treated with DEX showed inconsistent changes in infection intensity during the experiment (Fig. 3, right side). This result differs from that of Isobe and Lillehoj (1993) who showed in chickens that extended DEX treatment produced significantly higher numbers of total oocysts following primary and secondary experimental infections with coccidian *Eimeria mivati*. However, the results of these studies may not be directly comparable since we used a single DEX injection and monitored a chronic infection. In any case, it seems that the DEX treatment confounded the effects of carotenoids and PHA. This implies that the course of immune response to chronic infections is likely affected by glucocorticoids and the immunosuppressive effects of stress might confound the results of the studies trying to assess the role of carotenoids in immune response.

When the birds treated with DEX are excluded, focussing on the left side of the Fig. 3, a clear pattern emerges – immune stimulation by PHA suppresses the infection, but only among carotenoid-supplemented birds. We propose that suppression of coccidiosis by PHA

can be ascribed to the priming of the immune system. Such interpretation is supported by the evidence that immune cells (T-lymphocytes, macrophages and NK cells) and cytokines (IFN-γ, TNF-β and IL-2) involved in immune response against coccidiosis are also responsible for the delayed hypersensitivity reaction induced by PHA (Lillehoj, 1998). Additionally, PHA increases nitric oxide production (Sild and Hõrak, 2009), which is used to combat coccidiosis (Zhu et al., 2000).

The effects of PHA on coccidiosis emerged only among carotenoid-supplemented birds, which supports the concept of anti-parasitic effect of carotenoids (reviewed for example in McGraw et al., 2006). This finding is compatible to that of Baeta et al. (2008) who showed in blackbirds that carotenoid supplementation delayed the relapse of experimental infection. However, also in that study carotenoid supplementation did not affect the intensity of coccidiosis, which indicates that immunomodulatory effects of carotenoids are difficult to detect unless the dynamics of infection is carefully monitored. Yet another study on house sparrows found no effect of carotenoid supplementation on experimental coccidian infection; however carotenoids attenuated body mass decline in infected birds (Pap et al., 2009). Altogether these findings indicate that the effects of carotenoids on anti-parasite responses are subtle rather than robust and that their visualisation may require careful manipulations. For instance, in the current study we would not have detected the effect of carotenoids on coccidiosis without activating the immune system with PHA. Such complexity is perhaps not surprising, given that even in relatively simple models of immune stimulation with artificial antigens, carotenoids can elicit either pro-inflammatory (e.g., enhance PHA-induced swelling) or anti-inflammatory (e.g., Koutsos et al., 2006; Selvaraj and Klasing, 2006) effects, and that the direction of immunomodulation by carotenoids can depend on the level of dietary fat (Selvaraj et al., 2005). Further research on molecular mechanisms of carotenoid action on immune system is therefore warranted. The findings about involvement of carotenoids in modulation of an immune response against coccidiosis suggest that such research has a great potential in explaining why yellow, orange and red animal colouration has evolved.

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