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Immune-system activation depletes retinal carotenoids in house finches (*Carpodacus mexicanus*)

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SUMMARY

The costs of developing, maintaining, and activating the immune system have been cited as an important force shaping life-history evolution in animals. Immunological defenses require energy, nutrients and time that might otherwise be devoted to other life-history traits like sexual displays or reproduction. Carotenoid pigments in animals provide a unique opportunity to track the costs of immune activation, because they are diet-derived, modulate the immune system, and are used to develop colorful signals of quality. Carotenoids also accumulate in the retinas of birds, where they tune spectral sensitivity and provide photoprotection. If carotenoid accumulation in the retina follows the patterns of other tissues, then immune activation may deplete retinal carotenoid levels and impact visual health and function. To test this hypothesis, we challenged molting wild-caught captive house finches (*Carpodacus mexicanus*) with weekly injections of lipopolysaccharide (LPS) and phytohaemagglutinin (PHA) over the course of 8 weeks. Immunostimulated adult males and females produced significant antibody responses and molted more slowly than uninjected control birds. After 8 weeks, immune-challenged birds had significantly lower levels of specific retinal carotenoid types (galloxanthin and zeaxanthin), but there were no significant differences in the plasma, liver or feather carotenoid levels between the treatment groups. These results indicate that immune-system activation can specifically deplete retinal carotenoids, which may compromise visual health and performance and represent an additional somatic and behavioral cost of immunity.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/10/1709/DC1

Key words: lipopolysaccharide, phytohaemagglutinin, trade-off, vision.

INTRODUCTION

The ability of an individual to resist parasites and disease is essential for survival, but this resistance comes at a cost. Fever, phagocytosis, antibody production, and the entire spectrum of immune-system responses requires energy, nutrients, and time that could otherwise be devoted to other traits such as growth or reproduction (Klasing, 2004; Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002). Because these resource allocation patterns can have profound effects on individual fitness, the physiological costs of immune system function have come to be viewed as a key force shaping the evolution of life histories (Lochmiller and Deerenberg, 2000; Ricklefs and Wikelski, 2002; Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002). Consistent with this hypothesis, recent empirical studies have demonstrated the predicted costs of immune activation on self-maintenance, sexual attractiveness, reproductive output, and parental care (e.g. Bonneaud et al., 2003; Martin, 2005; Peters et al., 2004; Sanz et al., 2004). However, identifying specific mechanisms and resource currencies that link the immune system to life history traits has remained a challenge.

Carotenoid pigments are nutrient resources that have diverse biological functions and may provide a direct window into life-history trade-offs (Blount, 2004) as well as the costs of immune activity (Lozano, 1994; Møller et al., 2000; von Schantz et al., 1999). Animals cannot synthesize carotenoids *de novo*, but must acquire them from their diet through the direct or indirect consumption of plant material (Goodwin, 1984). Therefore, the supply of carotenoids to an animal may be limited by the environment and/or physiological uptake (Olson and Owens, 1998). Carotenoids can modulate immune function by stimulating T-cell and antibody production, facilitating gap-junction communication, influencing gene expression, and may also act as antioxidants, protecting vulnerable cells and tissues from reactive oxygen species generated during immune response (reviewed in Chew and Park, 2004). Carotenoids also generate sexually selected colors in many animals that can serve as reliable signals of individual health if carotenoids are traded off between coloration and immune function (Lozano, 1994; Møller et al., 2000; von Schantz et al., 1999). Consistent with this hypothesis, experimentally induced immune challenges have been shown to fade the carotenoid-based bare-part ornaments in a number of bird species [blackbirds, *Turdus merula* (Faivre et al., 2003); zebra finches, *Taeniopygia guttata* (McGraw and Ardia, 2003); mallard ducks, *Anas platyrhynchos* (Peters et al., 2004); and blue-footed boobies, *Sula nebouxii* (Torres and Velando, 2007)]. In the realm of behavioral ecology and ecophysiology, the immune, antioxidant and color signaling functions of carotenoids have received almost exclusive attention, but carotenoids also accumulate in the eyes of many animals where they provide photoprotection and facilitate visual function (Douglas and Marshall, 1999).

Carotenoids are found in the retinas of a diversity of vertebrates, including turtles, birds and primates (Douglas and Marshall, 1999) and have been extensively studied in humans, where accumulation is dependent upon dietary carotenoid intake, and linked to reduced risk of age-related macular degeneration (Seddon et al., 1994; Whitehead et al., 2006). In birds, retinal carotenoids are thought to have similar protective properties. For example, Thomson et al. (Thomson et al., 2002a; Thomson et al., 2002b) found that dietary supplementation increased retinal carotenoid levels and protected

Japanese quail (*Coturnix japonica*) against light-induced photoreceptor death. Retinal carotenoids also influence avian color vision by selectively filtering light reaching the photoreceptors to reduce overlap in the absorbance spectra of spectrally adjacent types, resulting in enhanced color discrimination and color constancy in variable lighting environments (Goldsmith and Butler, 2003; Vorobyev et al., 1998; Vorobyev, 2003). If immune system activity affects retinal carotenoid accumulation, it could impact visual health and performance.

The eye is extremely sensitive to immune activity because inflammation of ocular tissues can alter the precise spatial arrangement of elements within the eye [e.g. lens and photoreceptor mosaics (Streilein, 1999)] and thus compromise visual acuity. To counter these detrimental effects, the eye employs a complex of protective immunosuppressive mechanisms and is considered an 'immune privileged' tissue (Streilein, 1999). Despite these mechanisms, systemic immune challenges can still induce ocular inflammation (Bhattacherjee et al., 1983; Rosenbaum et al., 1980) and recently carotenoids have been identified as significant anti-inflammatory agents in the eye (Jin et al., 2006; Ohgami et al., 2003; Shiratori et al., 2005). Therefore immune activation may directly influence the accumulation of carotenoids in the eye.

The goal of our study was to examine if immune-system activation affects retinal carotenoid accumulation in the house finch (*Carpodacus mexicanus* Muller 1776), a passerine species whose carotenoid physiology and carotenoid-based color signaling have been investigated extensively (reviewed by Hill, 2002). Male house finches display carotenoid-based plumage coloration that varies from drab yellow to chromatic red, with females generally preferring the reddest males (Hill, 2002). Male coloration is dependent upon the metabolism and deposition of carotenoids into the feathers, and both dietary carotenoid intake and individual health influence coloration. Plumage redness is negatively correlated with the intensity of avian pox infection and feather mite parasitism (Thompson et al., 1997), and experimentally infecting molting birds with *Isospera* coccidians and *Mycoplasma gallisepticum* both result in reduced coloration of male plumage (Brawner et al., 2000; Hill et al., 2004). From these studies, we cannot separate the effect of immune-system activation from disease pathology, but they do suggest that immune challenges influence carotenoid allocation, metabolism, and/or deposition in feathers. It is not known in this or any other species how immune activity affects retinal carotenoid accumulation. In wild house finches, retinal carotenoid levels vary seasonally and are positively correlated with plumage coloration and body condition (Toomey and McGraw, 2009). Therefore, individual variation in immune activity and seasonal variation in disease prevalence (e.g. McClure, 1989) could be contributing to the observed variation in retinal carotenoid levels.

To examine the effects of immune-system activation on retinal carotenoid accumulation, we brought wild adult male and female house finches into captivity during the molt period (June–Sept), maintained them on a standardized diet, challenged a subset of these birds for 8 weeks with weekly injections of lipopolysaccharide and phytohaemagglutinin, then measured carotenoid levels in the plasma, liver, feathers and retina. We chose to use these two antigens over a long time course because the retina appears to be relatively stable to short-term perturbations. For example, we found that 8 weeks of dietary manipulation were required to cause a significant decline in retinal carotenoid levels in house finches, compared with the rapid depletion (within 2 weeks) from the plasma and liver (M.B.T. and K.J.McG., unpublished). The time course of the study is justifiable because long-term infections are common among wild house finches. The symptoms of *Mycoplasma gallisepticum* (MG) infection, which include extreme inflammation of the conjunctiva of the eye, last on average 10 weeks and up to 24 weeks in captive experimentally infected house finches (Kollias et al., 2004; Sydenstricker et al., 2006). Active infections of avian pox can last as long as 3 months (McClure, 1989).

MATERIALS AND METHODS

Bird capture and captive housing conditions

In May 2008, we captured 17 male and 19 female adult house finches in baited basket traps on the Arizona State University (ASU) campus in Tempe, Arizona (*sensu* McGraw et al., 2006). Birds were housed individually in wire cages ($0.6\,m \times 0.4\,m \times 0.3\,m$) in a greenhouse room on the ASU campus that allowed for natural light levels and day length. We fed finches a standard maintenance diet (ZuPreem small bird maintenance diet, Premium Nutritional Products Inc. Mission, KS, USA) and tap water *ad libitum* for the duration of the study. The maintenance diet contained two carotenoids: lutein ($1.15\pm0.12\,\mu g\,g^{-1}$) and zeaxanthin ($0.52\pm0.06\,\mu g\,g^{-1}$). These levels are relatively low compared with the gut contents of wild birds [mean $\approx 8\,\mu g\,g^{-1}$ total carotenoids (Hill et al., 2002)]; however, the circulating plasma carotenoid levels of our captive birds were equal to, or greater than, wild birds during the same period [mean $\approx 12\,\mu g\,ml^{-1}$ total carotenoids (McGraw et al., 2006)] indicating that our captive birds were not dietarily limited.

Antigens

To stimulate the immune system we used two antigens commonly used in eco-immunological studies with a broad range of effects on carotenoid physiology: lipopolysaccharide (LPS; from *S. typhimurium*, Sigma #L7261, St Louis, MO, USA) and phytohaemagglutinin (PHA; from *P. vulgaris*, Sigma #L8754). LPS is isolated from the cell wall of bacteria and inoculation simulates bacterial infection, stimulating both the innate and adaptive arms of the immune system (Klasing, 2004). Inoculation with LPS depletes circulating plasma carotenoid levels in chickens (*Gallus gallus*) (Koutsos et al., 2003), jungle fowl (McGraw and Klasing, 2006), and zebra finches (Alonso-Alvarez et al., 2004) and fades the carotenoid-based coloration of bare part ornaments in the blue-footed boobies (Torres and Velando, 2007) and zebra finches (Alonso-Alvarez et al., 2004). Systemic inoculation with LPS has also caused significant ocular inflammation in rats (Bhattacherjee et al., 1983; Rosenbaum et al., 1980). PHA is a mitogen that causes T cells to proliferate *in vitro* and results in the recruitment and activation of other leukocytes, resulting in edema (Martin et al., 2006). PHA-induced immune activity has been shown to reduce circulating carotenoid levels in blackbirds (*Turdus merula*) (Biard et al., 2009) and red-legged partridges (*Alectoris rufa*) (Perez-Rodriguez et al., 2008), increase circulating levels in Eurasian kestrels (*Falco tinnunculus*) (Costantini and Dell'omo, 2006), but to have no effect on plasma carotenoids in the greenfinches (*Carduelis chloris*) (Hõrak et al., 2007).

Experimental treatment

Beginning on 7 July 2008, we injected 10 randomly selected male and 10 randomly selected female birds intramuscularly (pectoralis major) with 0.1 mg of LPS and 0.1 mg of PHA dissolved in 0.05 ml of phosphate-buffered saline (PBS). These injections were repeated each week for 8 weeks. Seven male and nine female control birds did not receive injections of any kind. As in other studies (Adler et al., 2001), we omitted a sham injection treatment because sham injections can elicit an immune response, confounding our ability

to detect the effects of immune activation. To minimize differences in the levels of stress between the treatment and controls, all birds were housed, handled, and sampled such that the only difference between groups was the presence or absence of the injection. To track body condition, we weighed the birds (to the nearest 0.001 g) each week and recorded which primary feather was actively growing (1–9) on weeks 7 and 8. To track antibody titer and circulating carotenoid levels throughout the study, we collected a blood sample (~100 μl) from the ulnar vein on weeks 0, 2, 4 and 8 prior to administering the weekly antigen injection. These samples were immediately centrifuged (3 min at 7550 ***g***), and plasma was collected and stored at –80°C for up to 14 months prior to analysis. Unlike many previous studies utilizing PHA (e.g. Smits et al., 1999), we did not measure a swelling response because our injection site was not amenable to this assessment. One week after the final injection, all birds were killed and the retinas and liver tissue collected, using the method of Toomey and McGraw (Toomey and McGraw, 2009) and McGraw et al. (McGraw et al., 2006), respectively. By this time, all of the male birds had begun to molt into their ornamental plumage, but the rate and location of this molt varied among individuals; therefore we were not able to collect standardized reflectance measures for color analysis. We did, however, pluck 9–25 recently grown breast feathers from each male at the conclusion of the study to analyze feather carotenoid concentration (see below).

Carotenoid analyses

Carotenoid types and concentrations were determined using high-performance liquid chromatography (HPLC). Our HPLC system consists of a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA) with a Waters YMC carotenoid 5 μm column (4.6 mm×250 mm) heated to 30°C, and a Waters 2996 photodiode array detector. We used a constant solvent flow rate of 1.2 ml min^{-1}, but different three-step solvent gradients, to elute carotenoids particular to each tissue type (see below). We identified and quantified carotenoids by comparing their respective retention times and absorbance maxima (λ_{max}) to those of external standards [as in Toomey and McGraw (Toomey and McGraw, 2007; Toomey and McGraw, 2009)].

Plasma and liver carotenoids were extracted following McGraw et al. (McGraw et al., 2006) and eluted with a mobile phase solvent gradient of 42:42:16 (v/v/v) methanol:acetonitrile:dichloromethane for 11 min, then a linear gradient up to 42:23:35 (v/v/v) for 21 min, held isocratically at this condition until 25 min, and then returned to the initial condition from 25 to 30 min. Feather carotenoids were extracted by repeated (3×) grinding with a ball mill (MM200, Retsch GmbH and Co. KG, Haan, Germany) in 1 ml of methanol. These recently deposited feather carotenoids were highly esterified and required saponification for analysis in our HPLC system. To do this, we dried the feather extract under nitrogen, resuspended the pigment in 1 ml of 0.2 mol l^{-1} methanolic KOH, capped the solution under nitrogen, and held the samples in the dark at room temperature for 6 h. We then added 1 ml of saturated NaCl and 2 ml of distilled H_2O, and vigorously shook the samples with each addition. To re-extract carotenoids from this solution, we then added 3 ml of hexane:*tert*-butyl methyl ether (1:1) to each tube, shook them for 1 min to mix the layers thoroughly, centrifuged the samples for 5 min at 1500 ***g***, and collected the supernatant. These saponified feather extracts (containing xanthophylls, based on the xanthophyll diet they were fed) (Inouye et al., 2001) were then analyzed under the same HPLC conditions as were the plasma and liver samples.

We extracted carotenoids from whole retinas following the method of Toomey and McGraw (Toomey and McGraw, 2009) and used a two-step saponification procedure to ensure reliable quantification of the entire suite of retinal carotenoids (for details, see Toomey and McGraw, 2007). Retinal carotenoids were eluted with a mobile phase solvent gradient of 44:44:12 (v/v/v) methanol:acetonitrile:dichloromethane for 11 min, then a linear gradient up to 42:23:35 (v/v/v) by 21 min, held isocratically at this condition until 25 min, and then returned to the initial condition from 25 to 30 min. Retinal carotenoid concentrations are presented as micrograms of carotenoid per whole retina [as in Toomey and McGraw (Toomey and McGraw, 2007; Toomey and McGraw, 2009)].

Carotenoid types and distribution

Our captive birds contained carotenoid types and concentrations in plasma and liver that were consistent with observations from wild house finches. Within each tissue, the concentrations of different carotenoid types were significantly positively intercorrelated; therefore, we used total plasma carotenoid concentration for all subsequent analyses (supplementary material Table S1) (McGraw et al., 2006; Toomey and McGraw, 2009). The recently grown feathers of our captive male house finches contained canary xanthophylls A and B, lutein, zeaxanthin, and β-cryptoxanthin (supplementary material Table S2). Owing to the diet they received, and as commonly occurs with captive molting house finches (Hill, 2002), these feathers were yellow and lacked any traces of carotenoids responsible for the production of red color (e.g. astaxanthin, canthaxathin, echinenone, 3-hydroxyechinenone, adonirubin, and 4-oxo-rubixanthin) (Inouye et al., 2001). Concentrations of all feather carotenoids were significantly positively intercorrelated (supplementary material Table S2), and we used total feather carotenoid concentration for all subsequent analyses. Consistent with wild birds, the retinas of our captive birds contained six major types of carotenoids (Table 1) (Toomey and

Table 1. Concentration of and Pearson's correlations between retinal carotenoid types for treatment and control groups combined

		Pearson's correlation (r_P)				
	Concentration (μg retina^{-1})	Astaxanthin	Galloxanthin	Lutein	Zeaxanthin	Unknown
Astaxanthin	0.47±0.022	–				
Galloxanthin	0.42±0.017	0.27	–			
Lutein	0.065±0.0031	–0.033	**0.37**	–		
Zeaxanthin	0.093±0.0036	0.26	**0.65**	0.16	–	
Unknown	0.037±0.0010	0.17	**0.61**	0.22	**0.93**	–
ε-Carotene	0.015±0.000082	–0.08	**0.45**	0.079	**0.44**	**0.48**

Bold type indicates significant correlations ($P \leq 0.007$). For detailed descriptions of the spectral properties and HPLC retention times of these carotenoids see Toomey and McGraw (Toomey and McGraw, 2009).

McGraw, 2009). The concentrations of some, but not all, retinal carotenoid types were significantly positively intercorrelated (Table 1). However, because the accumulation of the different retinal carotenoid types are photoreceptor specific (Goldsmith and Butler, 2003; Goldsmith et al., 1984) and differentially affected by dietary carotenoid manipulations (Bhosale et al., 2007; Scheidt et al., 1991), we statistically analyzed each of the retinal carotenoid types separately.

Antibody titer measurement

We measured antibody response to LPS and PHA to quantify the immunostimulatory effects of each antigen. This method differs from many previous eco-immunology studies (e.g. Smits et al., 1999) that combine multiple measures of immunocompetence into a single metric (e.g. adaptive and innate immunity within a PHA-induced swelling response) (Martin et al., 2006), and provides the advantage of directly measuring the immunogenicity for both antigens in a single type of assay. To measure antibody response we used an enzyme-linked immunosorbant assay (ELISA) (*sensu* Butler and Dufty, 2007). First, we incubated 96-well plates (Fisher 468667, Hampton, NH, USA) at 4°C overnight with $0.5\,\mathrm{mg\,ml^{-1}}$ LPS or $0.5\,\mathrm{mg\,ml^{-1}}$ PHA suspended in sodium carbonate (pH 9.6) buffer. We then washed the wells with PBS and Tween 20 (0.05% by volume) and blocked the wells with blocking buffer (PBS with 1% bovine serum albumin and 0.1% sodium azide) at room temperature for 2 h. We washed the plates three times then added 100 μl of plasma diluted 1:12,800 in blocking buffer, and incubated overnight at 4°C. Plasma dilutions for both antibodies (LPS and PHA) were previously determined in a separate assay to ensure that concentrations of antibody fell within the linear range of the assay. We then washed the plates, added 80 μl of HRP-labeled secondary anti-avian IgG antibody (A140-110P; Bethyl Laboratories, Montgomery, TX, USA) diluted 1:10,000 in blocking buffer, and incubated at room temperature for 2 h. After washing the plates, we added 100 μl of tetramethylbenzidine (TMB). We stored the plates at room temperature, protected from light, for 20 min and then added 50 μl of H_2SO_4. Within 10 min, we measured the absorbance of the contents of each well at a wavelength of 450 nm using SOFTmax PRO (Molecular Devices, CA, USA). Absorbance values were averaged across triplicate samples (intra-assay variation ranged from 3.07 to 4.41) and used in all subsequent analyses.

Statistical analyses

We analyzed temporal changes in antibody titer, body mass, molt progress and plasma carotenoid levels between treatment groups with repeated-measures analyses of variance (RM ANOVA), with sex and treatment (control *versus* challenged) as the between-subjects factors. There were no significant interactions between the between-subjects factors, so we omitted these from final models. We used univariate analysis of variance (ANOVA) to examine the effects of experimental treatment on feather, liver and retinal carotenoid levels measured at the conclusion of the study. Sex and treatment were the independent factors in analyses, and there were no significant interactions between these terms so we omitted these from our final ANOVA models. Because retinal carotenoid levels differed significantly between treatments (see below), we examined how the extent of immune response among challenged birds related to retinal accumulation with an additional analysis of covariance (ANCOVA) for the immune-challenged birds only, with sex as the independent factor and the maximum LPS and PHA titer as covariates and retinal carotenoid levels as dependent variables. We examined the residuals of the ANOVA models and found no major deviations from normality or equal variance.

RESULTS

Response to immune challenge

As expected, immunization with LPS and PHA caused a significant increase in circulating antibodies to each of these antigens (Fig. 1; LPS–week: $F_{3,54}$=27.66, P<0.001; PHA–week: $F_{3,54}$=12.31, P<0.0001). However, the temporal and sex-specific effects of the immune-system treatment differed for these two antigens. LPS antibody titer increased significantly between weeks 0, 2 and 4 (*post-hoc* Bonferroni, P≤0.03), but did not differ significantly between weeks 4 and 8. Male finches had a significantly stronger antibody response to LPS than did female finches (Fig. 1A; ANOVA, sex: $F_{1,18}$=6.61; P=0.018). PHA antibody levels increased significantly from week 0 to 2 (*post-hoc* Bonferroni, P=0.001), with a non-significant decline throughout the remainder of the study (Fig. 1B). The maximum measured LPS and PHA antibody titers were marginally positively intercorrelated in immune-challenged male finches (r_P=0.62, P=0.058), but not females (r_P=0.19, P=0.60).

Effects of immune challenge on body mass and molt progress

There was no significant effect of immune challenge on body mass (RM ANOVA – week×treatment: $F_{1.92,63.51}$=1.46, P=0.24). The mass of both challenged and control birds significantly increased between weeks 2, 4 and 8 (Fig. 2A; RM ANOVA week×week: $F_{1.92,63.51}$=13.02, P<0.0001; *post-hoc* Bonferroni, P<0.0001). In week 7, immune-challenged birds had molted significantly fewer primary feathers than controls (Fig. 2B; ANOVA treatment: $F_{1,33}$=4.71; P=0.037); however, in week 8 the treatment groups converged in molt progress (Fig. 2B; ANOVA treatment: $F_{1,33}$=0.40; P=0.53).

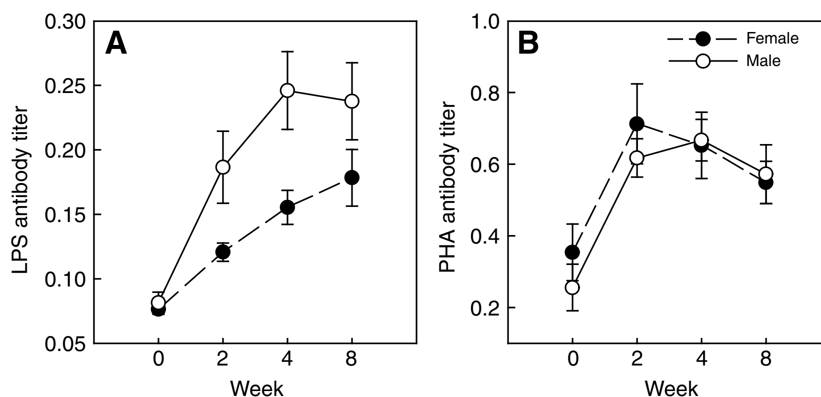

Fig. 1. Response to immune challenge. Antibody titer of (A) lipopolysaccharide (LPS) and (B) phytohaemagglutinin (PHA; measured as optical density at 450 nm) of immune-challenged house finches by sex before injection (week 0) and weeks 2, 4 and 8 of the study. Values are means ± s.e.m.

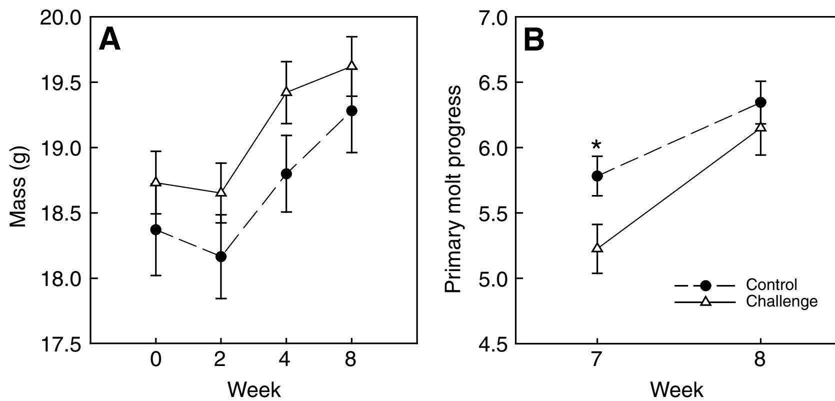

Fig. 2. Effects of immune challenge on body mass and molt progress. (A) Body mass of immune-challenged (weekly with LPS and PHA) and uninjected control house finches before injection (week 0) and at weeks 2, 4 and 8 of the study. (B) Number of molted primary feathers for immune-challenged control birds on weeks 7 and 8 of the study. *Significant difference between challenged and control groups (see text). Values are means ± s.e.m.

Effects of immune challenge on carotenoid accumulation

Immune challenge significantly depleted retinal carotenoid concentrations, and these changes were specific to retinal carotenoid types. Injected birds had significantly lower levels of retinal galloxanthin, zeaxanthin and an unknown carotenoid than control birds (Fig. 3, Table 2), but there were no significant differences in astaxanthin, lutein or ε-carotene levels (Table 2). Among challenged birds, retinal carotenoid levels of any type were not significantly related to maximum LPS or PHA antibody titers (P>0.18). Throughout the study, there were no significant differences in total plasma carotenoid levels between challenged and control birds (RM ANOVA, week×treatment: $F_{2.49,79.59}$=0.28; P=0.80); however, levels differed significantly among weeks, with the highest levels occurring in week 8 of the study (Fig. 4A; RM ANOVA, week: $F_{2.49,79.59}$=10.68; P<0.0001). From liver samples collected at the conclusion of the experiment, we found no significant differences in total liver carotenoid concentrations between treatment groups (ANOVA treatment: $F_{1,33}$=0.09; P=0.77; Fig. 4B). The carotenoid concentration in growing ornamental breast feathers did not differ between challenged and control male house finches (t=–1.013; d.f.=15; P=0.33, Fig. 4B).

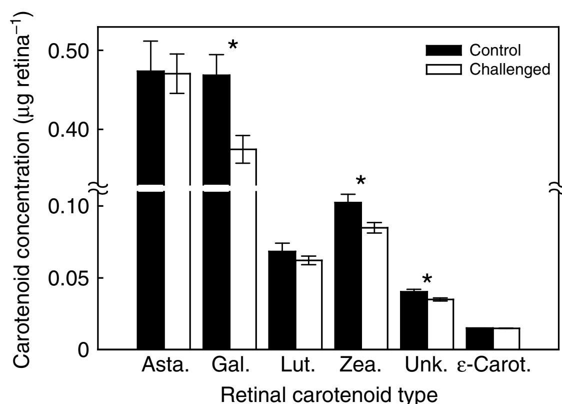

Fig. 3. Effects of immune challenge on carotenoid accumulation. Concentration of each retinal carotenoid type for immune challenged (weekly for 8 weeks with LPS and PHA) and uninjected control house finches measured at the conclusion of the study. *Significant difference between challenged and control birds (Table 2). Asta., astaxanthin; Gal., galloxanthin; Lut., lutein; Zea., zeaxanthin; Unk., unknown; ε-Carot., ε-carotene. Values are means ± s.e.m.

DISCUSSION

We administered a sustained immune-system challenge to captive molting house finches to determine its effect on carotenoid allocation to retinal tissue. Our weekly LPS and PHA injections successfully elevated circulating levels of LPS- and PHA-specific antibodies. Prolonged immune-system activation also had other notable effects on finch physiology and morphology, namely by slowing molt progress and thus presumably limiting resources available for feather growth. This is consistent with trade-offs between immune response and feather growth observed in house sparrows (*Passer domesticus*) (Martin, 2005) and pied flycatchers (*Ficedula hypoleuca*) (Sanz et al., 2004). Although one might expect a costly immune response to cause a loss of body mass, we found no such effect. However, a number of other studies have reported significant changes in carotenoid physiology in response to experimentally induced immune activity with no significant changes in body mass (e.g. Alonso-Alvarez et al., 2004; Biard et al., 2009; Hõrak et al., 2006; Perez-Rodriguez et al., 2008; Peters et al., 2004).

Our prolonged immune-system challenge significantly depleted retinal carotenoid levels of our captive house finches. This is the first study to show an association between an animal's immune-system activity and retinal carotenoid accumulation. One possible concern when interpreting our results is that the level and duration of immune stimulation in our experiment was much greater than that experienced by finches in nature. However, several lines of evidence suggest that our immune challenge was not an unnaturally stressful manipulation: (1) birds maintained and even increase in body mass over the course of the study; (2) although slowed in the challenged birds, molt progressed within the natural time frame for the house finch (Hill, 1993); (3) the carotenoid levels in tissues known to be sensitive to immune challenge (e.g. plasma) were not

Table 2. Results of ANOVAs for retinal carotenoid concentration in relation to immune challenge and sex

	Treatment		Sex	
	F	P	F	P
Astaxanthin	0.003	0.96	0.041	0.84
Galloxanthin	**9.055**	**0.005**	0.046	0.83
Lutein	1.29	0.26	3.15	0.085
Zeaxanthin	**6.68**	**0.014**	0.41	0.53
Unknown	**7.74**	**0.009**	0.002	0.96
ε-Carotene	0.63	0.43	2.39	0.13

The non-significant treatment × sex interaction term was omitted from the model. d.f.=1,33 for all comparisons.

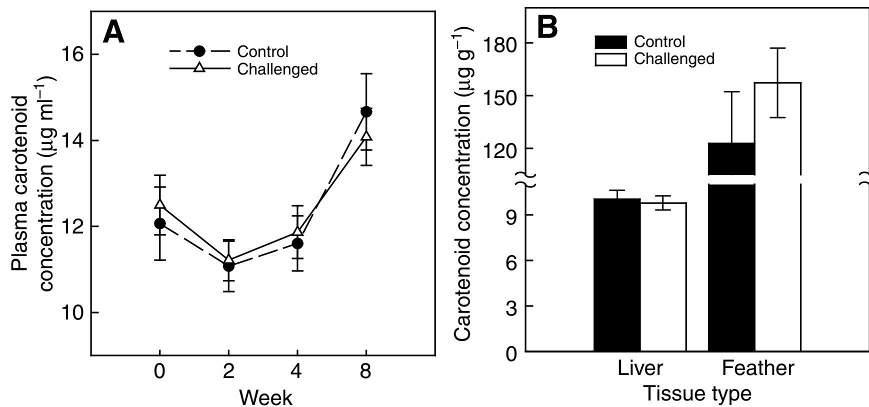

Fig. 4. Effects of immune challenge on carotenoid accumulation. (A) Plasma carotenoid concentrations of immune-challenged (weekly with LPS and PHA) and uninjected control house finches before injection (week 0) and at weeks 2, 4 and 8 of the study. (B) Liver tissue carotenoid concentration of control and immune-challenged birds of both sexes and the carotenoid concentrations of male ornamental breast feathers from control and challenged male birds at the conclusion of the study. Values are means ± s.e.m.

affected by our manipulation, and (4) our 8-week manipulation falls well within the natural duration of common infections such as *Mycoplasma gallisepticum* and avian pox (see Introduction). Therefore, our manipulation is probably representative of challenges birds may face in the wild.

Carotenoid depletion was specific to the retina and we found no significant effect of immune system activation on plasma, liver and feather carotenoid levels. This result contrasts with a number of previous studies that have shown significant declines in carotenoid levels in the blood and colorful ornaments following an immune challenge (e.g. Biard et al., 2009; Faivre et al., 2003; Peters et al., 2004; Perez-Rodriguez et al., 2008; McGraw and Klasing, 2006). However, Biard et al. (Biard et al., 2009) recently determined that the depletion of circulating carotenoids during a PHA-induced immune response in blackbirds is relatively short-lived and the effects disappear after >2 days. Therefore, transient depletion of plasma carotenoids may have occurred in our immune-challenged birds, but we were not able to detect it because our sampling occurred 7 days after the injections. The lack of an effect of immune challenge on feather carotenoid accumulation is consistent with studies of goldfinches (*Carduelis tristis*) (Navara and Hill, 2003) and great tits (*Parus major*) (Fitze et al., 2007) and suggests that feather carotenoid deposition may be somehow buffered against the relatively rapid changes in carotenoid availability and allocation. However, because of the limited concentrations and types of dietary carotenoids available in our study, which induced all males to grow pale yellow feathers, we must cautiously interpret this result and perform follow-up studies using proper metabolic precursors (e.g. β-cryptoxanthin) (McGraw et al., 2006) that allow development of sexually preferred, elaborate red plumage coloration.

The fact that immunological effects on carotenoid accumulation were retina-specific suggests that we isolated a particularly strong stressor for the eye and its immunomodulatory components. Generally, retinal carotenoids are considered relatively stable when compared with other tissues (e.g. Wang et al., 2007), but may be particularly affected by immune challenges because the eye exhibits a high level of immunosuppression to counter the detrimental effects of inflammation (see Introduction). Systemic immune challenges with LPS are known to cause significant inflammation, and nitric oxide (a damaging pro-oxidant) production in the rat eye (Bhattacherjee et al., 1983; Goureau et al., 1995; Rosenbaum et al., 1980). Carotenoid supplementation has been shown to reduce inflammation and inhibit the production of nitric oxide in the rat eye following LPS challenge (Jin et al., 2006; Ohgami et al., 2003; Shiratori et al., 2005). Therefore, we may have seen a decline in retinal carotenoid levels because these pigments were being allocated to and consumed by anti-inflammatory and anti-oxidant functions in the eye. However, in birds, the immunosuppressive properties of the eye and the fine-scale molecular relationships between immune activation and carotenoid physiology have yet to be explored. Regardless of the mechanism, our results do suggest that long-term health perturbations represent significant costs toward producing or maintaining carotenoid pigments in retina.

Not only were the effects of immune challenges retina specific, but within the retina only certain types of carotenoids were affected by our treatment. Retinal galloxanthin, zeaxanthin and an unknown carotenoid were significantly depleted in the immune-challenged birds, whereas the concentrations of other carotenoid types remained unchanged. These carotenoid-specific changes may be related to differences in accumulation mechanisms and turnover rates among retinal carotenoid types. In wild house finches, retinal levels of the carotenoids that were immune-treatment-sensitive in this study (e.g. galloxanthin and zeaxanthin) were significantly correlated with circulating plasma carotenoid levels, while retinal astxanthin and ε-carotene levels, which were unaffected by our immune challenge, did not significantly correlate with plasma carotenoids (Toomey and McGraw, 2009). This suggests that concentrations of some retinal carotenoid types may be maintained through replenishment from the circulating pool, while other types are buffered against short-term changes and turn over relatively slowly. Further support for this idea comes from Bhosale et al. (Bhosale et al., 2007), who tracked the accumulation of isotopically labeled dietary zeaxanthin in quail retina and found that the degree of labeling differed among retinal carotenoid types. After 16 weeks on the labeled diet, zeaxanthin and galloxanthin showed high levels of labeling (94% and 58%, respectively), whereas retinal astaxanthin was relatively low (28%) (Bhosale et al., 2007).

The specificity of these changes is particularly interesting because these retinal carotenoids differ not only in their patterns of accumulation, but have distinct functions in the avian eye. Retinal carotenoids in birds accumulate in colorful oil droplets located between the inner and outer segments of the cone photoreceptors. Each of the five cone photoreceptor types contain a different colored oil droplet containing specific types of carotenoids. For example, the long-wavelength-sensitive cone contains a red oil droplet pigmented with astaxanthin, whereas the medium-wavelength-sensitive cone has a yellow oil droplet pigmented with zeaxanthin (Goldsmith et al., 1984; Goldsmith and Butler, 2003). These oil droplets filter light that reaches the retina, not only providing photoprotection, but also enhancing color vision. Behavioral studies of domesticated birds suggest that the complete exclusion of carotenoids from the avian retina can shift color preferences and

spectral sensitivity (Bowmaker et al., 1993; Duecker and Schulze, 1977; Wallman, 1979). A recent modeling study by Lind and Kelber (Lind and Kelber, 2009) indicates that even subtle shifts (10 nm) in retinal oil droplet filtering can significantly change predicted color matches and chromatic contrast values within a given visual system. Carotenoids are also important photoprotectants and, in quail, increased retinal zeaxanthin accumulation prevents light-induced photoreceptor death (Thomson et al., 2002a; Thomson et al., 2002b). Therefore, immune system activity induced depletion of retinal carotenoids could have direct impacts on both visual health and function.

Studies of carotenoids and life-history evolution (Blount, 2004) have primarily traced pigment allocation between two competing functions – health and ornamentation – to identify the information content and honesty-maintaining mechanisms of sexual signals (Lozano, 1994; Møller et al., 2000; von Schantz et al., 1999). Our results indicate that immune-system activation may also divert carotenoid resources away from the visual systems that receive these colorful signals. If retinal carotenoid depletion brought about by immunological challenge compromises the health and performance of the eye, then this represents a previously unappreciated carotenoid-mediated cost of immune activity. Moreover, the reliability with which visual signals are received and perceived may differ with a receiver's health status. Sensory phenotype has been implicated as an important factor shaping individual preference functions in the context of mating (Widemo and Sæther, 1999), introducing the possibility of a common physiological mechanism (carotenoid allocation/accumulation) mediating both a sexual trait (colorful plumage) and preference.

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Table S1. Concentrations of and Pearson's correlations among plasma carotenoid types for treatment and control groups combined

		Pearson's correlation (r_P)	
Carotenoid	Concentration (μg ml^{-1})	Lutein	Zeaxanthin
Lutein	8.56±0.20	–	
Zeaxanthin	3.13±0.07	0.95	–
β-Cryptoxanthin	0.71±0.08	0.39	0.44

Values are mean±s.e.m.

The concentrations of all carotenoid types were significantly inter-correlated ($P \leq 0.018$).

Table S2. Concentrations of and Pearson's correlations among feather carotenoid types for treatment and control groups combined

		Pearson's correlation (r_P)		
Carotenoid	Concentration ($\mu g\ g^{-1}$)	Canary xanthophyll A and B	Lutein	Zeaxanthin
Canary xanthophyll A and B	35.77±4.71	–		
Lutein	61.11±7.60	0.58	–	
Zeaxanthin	38.45±3.89	0.66	0.95	–
β-Cryptoxanthin	9.84±1.18	0.77	0.77	0.77

Values are mean±s.e.m.

The concentrations of all carotenoid types were significantly inter-correlated ($P \leq 0.014$).