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Carotenoids bolster immunity during moult in a wild songbird with sexually selected plumage coloration

KEVIN J. MCGRAW[1]*, PAUL M. NOLAN[1,2] and ONDI L. CRINO[1,3]

[1]*School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA*
[2]*Department of Biology, The Citadel, Charleston, SC 29409, USA*
[3]*Division of Biological Sciences, University of Montana, Missoula, MT 59812, USA*

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Carotenoid-based colours in animals are valuable models for testing theories of sexual selection and life-history trade-offs because the pigments used in coloration are chemically tractable in the diet and in the body, where they serve multiple purposes (e.g. health enhancement, photoprotection). An important assumption underlying the hypothesized signalling value of carotenoid coloration is that there is a trade-off in carotenoid pigment allocation, such that not all individuals can meet the physiological/morphological demands for carotenoids (i.e. carotenoids are limited) and that only those who have abundant supplies or fewer demands become the most colourful. Studies of carotenoid trade-offs in colourful animals have been limited largely to domesticated species, which may have undergone artificial selection that changed the historical/natural immunomodulatory roles of carotenoids, to young animals lacking carotenoid-based signals or to species displaying carotenoid-based skin and bare parts. We studied the health benefits of carotenoids during moult in house finches (*Carpodacus mexicanus*), which display sexually selected, carotenoid-based plumage coloration. We manipulated dietary carotenoid availability during both winter (nonmoult) and autumn (moult) in captive males and females and found that carotenoid-supplemented birds mounted stronger immune responses (to phytohemagglutinin injection and to a bacterial inoculation in blood) than control birds only during moult. This study provides experimental, seasonal support for a fundamental tenet of Lozano's 'carotenoid trade-off' hypothesis and adds to a growing list of animal species that benefit immunologically from ingesting higher dietary carotenoid levels.

ADDITIONAL KEYWORDS: carotenoid pigmentation – *Carpodacus mexicanus* – house finch – immunocompetence – trade-off.

INTRODUCTION

A variety of animal traits, including weaponry, elaborate vocalizations, and complex courtship displays, serve as honest, 'condition-dependent' indicators of mate attractiveness or competitive ability (Maynard-Smith & Harper, 2003). Behavioural ecologists have shown special interest of late in the proximate mechanisms that keep such signals honest (Searcy & Nowicki, 2005). Factors such as nutrition, health, and reproductive/energetic expenses are typically considered to be limiting for maximal expression of such traits (Griffith, Parker & Olson, 2006).

For many animal signals, it is not yet apparent how particular dietary, immunological or energy perturbations influence trait development (Searcy & Nowicki, 2005). Others, however, present a quite tractable system (or 'currency') for quantifying units of signal expression and testing theories of honesty enforcement. One such example is the ornamental carotenoid coloration displayed by many birds and fish (McGraw, 2006). The carotenoid pigments used for coloration can be chemically traced from the diet, from which they must be acquired, and in the body, where they serve multiple functions (e.g. immunomodulation, defence from solar radiation). Because of their many utilities, these colours have also emerged as ideal models for testing concepts in sexual selection in the context of life-history trade-offs (Blount, 2004).

*Corresponding author. E-mail: kevin.mcgraw@asu.edu

Detailed studies of carotenoid pigmentation in animals such as guppies (*Poecilia reticulata*) and house finches (*Carpodacus mexicanus*) reveal that this form of coloration can be a reliable signal of dietary access to carotenoids (Grether, Hudon & Millie, 1999; Hill, Inouye & Montgomerie, 2002), general nutritional/energy state (Nicoletto, 1991; Frischknecht, 1993; Hill, 2000), and current health state (Houde & Torio, 1992; Brawner, Hill & Sundermann, 2000; Horak *et al*., 2004). Two main production costs underlying signal honesty have been proposed: (1) carotenoids are limiting nutrients in the environment (Endler, 1983; for which there is now some support; see above) and (2) carotenoids are valuable antioxidants and modulators of immunity (Burton & Ingold, 1984). The latter observation led Lozano (1994) to advance his seminal 'carotenoid trade-off' hypothesis, namely that carotenoid-coloured animals are faced with the trade-off of allocating carotenoids to health versus coloration, and that only those animals with high carotenoid supplies can meet the demands of allocating pigments to both health and coloration.

In recent years, this hypothesis has attracted much empirical and conceptual attention in the literature (Shykoff & Widmer, 1996; Hartley & Kennedy, 2004; van Der Veen, 2005; Perez-Rodriguez, 2009). A major thrust in this research and debate initially was whether individuals from any colourful species are ever limited in quantities of carotenoids needed to bolster immunity (Hill, 1999; Lozano, 2001). Subsequently, carotenoids have been found to enhance immunity in animals with carotenoid-based sexual colours (Blount *et al*., 2003; Grether *et al*., 2004) and evidence for a carotenoid trade-off between self-maintenance and coloration functions has emerged from several field and laboratory studies of various bird and fish species (Aguilera & Amat, 2007; Clotfelter, Ardia & McGraw, 2007; Alonso-Alvarez *et al*., 2008; Baeta *et al*., 2008).

However, not all studies support the carotenoid trade-off hypothesis (Navara & Hill, 2003; Fitze *et al*., 2007; Costantini & Møller, 2008; Lin *et al*., 2010) and there are some taxonomic and logistical biases in previous work that may hinder our ability to draw broad conclusions. Studies largely have been conducted either on: (1) domesticated species (McGraw & Ardia, 2003; Alonso-Alvarez *et al*., 2004), which have been subjected to a history of inbreeding and for which carotenoids may unnaturally boost health because of their artificially compromised immune system; (2) young birds, which lack adult carotenoid ornamentation (Fenoglio, Cucco & Malacarne, 2002; Saino *et al*., 2003) or (3) animals with carotenoid-based bare parts (e.g. skin, scales, beak; Faivre *et al*., 2003; McGraw & Ardia, 2003; Peters *et al*., 2004; Perez-Rodriguez *et al*., 2008) that can change colour rapidly (Rosen & Tarvin, 2006; Velando, Beamonte-Barrientos & Torres, 2006). By contrast to these emphases, much of the past work on carotenoid-based sexual signals has been performed on adults from free-living bird species with colourful plumage (Johnson, Rosetta & Burley, 1993; Pryke, Lawes & Andersson, 2001; Hill, 2002).

Support for the 'carotenoid trade-off' hypothesis has largely come from observations that (1) providing supplemental carotenoids to animals boosts their health state or (2) carotenoid pigments or coloration decline in the face of an immune challenge or oxidative stress (citations above). However, demonstrating a true molecule-specific trade-off under these circumstances is not trivial (Biard *et al*., 2009), especially when the tissue fate of pigments is not tracked (i.e. using isotope labelling) or when other processes that could be impacted by health state, such as food intake or pigment metabolism, are not monitored. Clearly, more quantitative and comparative approaches are needed to further test this 'carotenoid trade-off' hypothesis, particularly for avian plumage signals. At present, there is only mixed support for this idea in wild birds with sexually selected, carotenoid-pigmented plumage. Navara & Hill (2003) found no immunostimulatory role for xanthophyll carotenoids in male American goldfinches (*Carduelis tristis*) during feather growth, whereas Aguilera & Amat (2007) found that carotenoids enhanced immunity in male greenfinches (*Carduelis chloris*) that had just completed moult.

One prediction that emerges from the 'carotenoid trade-off' hypothesis for birds with colourful plumage is that carotenoids may only be differentially limiting or beneficial at the time of year when pigments are actively being used for coloration (e.g. during moult). In other words, carotenoid allocation to feathers may render immune systems deficient in and more likely to be aided by increased carotenoid supplies compared to other times of year. The fact that annual circulating levels of carotenoids in wild house finches are lowest during moult also suggests greatest carotenoid limitations at this time (Toomey & McGraw, 2009). Hence, a test of seasonal variation in carotenoid immunomodulation may be informative for understanding how and when carotenoids may play valuable, limiting immunomodulatory roles.

In the present study, we experimentally tested the relationship between carotenoids and immunity in nonmoulting and moulting captive house finches (*Carpodacus mexicanus*). The variable red-to-yellow, carotenoid-based plumage of male house finches has served as a classic example of an honest signal of quality in animals (Hill, 2002). Surprisingly, despite all of the studies that have been performed on diet,

health, and coloration in house finches, including one in which males with redder plumage were found to show higher disease clearance rates (Hill & Farmer, 2005), no information is yet available on the relationship between systemic carotenoids and health *per se* in this species. At two different times of year (winter and autumn), we manipulated naturally available xanthophyll carotenoids in the diet and examined their effect on two indices of immunocompetence (bactericidal potency of whole blood and swelling response to a mitogen injection). We selected these two measures of immunity because recent work in chickens (*Gallus gallus domesticus*; Selvaraj *et al*., 2006) and other avian species (see below) suggests that they are especially sensitive to carotenoid status. The fact that we studied both males and females allowed us to test the possibility that males, who deposit more carotenoids into plumage than females and thus may be more carotenoid-limited (for a given dietary intake), would experience greater health benefits when given supplemental carotenoids during moult compared to females.

MATERIAL AND METHODS

General capture and treatment of birds

From 4–11 October 2004, 16 male and 16 female house finches in adult plumage were captured in basket traps at established sunflower-seed feeder stations on the campus of Arizona State University. Unlike the majority of work carried out on house finches (from their introduced range in the eastern United States), these birds were captured from their native range, in the desert southwestern USA (Hill *et al*., 2002; Deviche & Cortez, 2005; Toomey & McGraw, 2009). At capture, we banded each bird with a uniquely numbered metal leg ring, measured body mass to the nearest 0.01 g with an electronic balance, and scored plumage coloration using a Colortron II reflectance spectrophotometer (Hill, 1998). Hue and saturation were used as our measures of carotenoid coloration because they are the most variable, sexually valuable, and repeatably quantifiable tristimulus colour scores in this species; we averaged hue and saturation scores from duplicate readings taken on the rump in females and on the crown, breast, and rump in males to compute mean values for each animal (Hill, 2002).

For the duration of the captive study, birds were housed in an Institutional Animal Care and Use Committee approved, climate-controlled greenhouse room, under natural light conditions, and in male–female pairs within small wire cages (for housing details, see McGraw, 2005). We fed them a base, *ad libitum* diet of tap water and ZuPreem® AvianMaintenance™ Natural Premium Diet for Canaries and Finches (Premium Nutritional Products Inc.). This pelleted diet contains a xanthophyll carotenoid content of 8.5 $\mu g\ g^{-1}$ (McGraw, 2005) and minor amounts of β-cryptoxanthin and β-carotene (< 1 $\mu g\ g^{-1}$; K. J. McGraw, unpubl. data). This represents just less than an average dietary carotenoid dose for moulting house finches, which acquire a mean of 12 $\mu g\ g^{-1}$ total carotenoids from their food (range 0.5–80 $\mu g\ g^{-1}$) (Hill *et al*., 2002).

Captive dietary carotenoid supplementation experiments

Birds were allowed an initial 6–7 weeks (depending on their capture date) to acclimate to the captive environment. Then, we randomly divided the birds into two groups of eight females and eight males each. Identical eight-week feeding experiments were then run in the winter (23 November 2004 to 20 January 2005) and the following autumn (26 July to 20 September 2005). One group (control birds) continued to consume the base food/water diet, whereas we added 0.158 g of lutein beadlets and 0.028 g of zeaxanthin beadlets to each litre of drinking water for the other group (carotenoid-supplemented birds), which approximates the lutein : zeaxanthin ratio found in the plasma of moulting birds at our study site (McGraw, Nolan & Crino, 2006a). This resulted in a supplemental dose in the drinking water (9.4 $\mu g\ g^{-1}$) that matched the total carotenoid concentration that treatment birds were receiving in their food. It is important to note that birds remained in the same treatment group during each experiment but, between experiments (20 January to 25 July), all birds were returned to the base, low-carotenoid diet. We did not find carry-over effects of the first (winter) feeding period on health or carotenoid status at the start of the second (moult) experiment (see Results below).

On the day before beginning each experiment, we weighed each bird as above and drew blood (approximately 150 μL from the alar vein) to determine pre-experimental plasma carotenoid levels as well as the bactericidal potency of whole blood (for procedures, see below). The latter is a newly emerging immune assay in vertebrates (Tieleman *et al*., 2005; Matson, Tieleman & Klasing, 2006) and one previously used by us to probe (and confirm) the immune-enhancing properties of carotenoids in two avian species: red junglefowl (*Gallus gallus*; McGraw & Klasing, 2006) and society finch (*Lonchura domestica*; McGraw *et al*., 2006b). This *in vitro* method explores the extent to which constitutive, innate immune mechanisms (e.g. complement, leukocytes) in blood can kill *Escherichia coli* bacteria.

Then, 7.5 weeks into the 8-week supplementation period, we again weighed birds and drew blood for plasma-carotenoid and bacterial-killing assays. In the autumn experiment, this was carried out at a time (in mid-September) when birds had completed 64 ± 7% (mean ± SE) of colourful plumage moult (range 15–90%). On the final 2 days of carotenoid supplementation, we completed our second assay of health, comprising a commonly used mitogenic challenge with phytohemagglutinin (PHA) that measures inducible adaptive immunity (Smits, Bortolotti & Tella, 1999). We and others have used this technique previously to demonstrate carotenoid facilitation of the PHA response (thickness of wing-web swelling) in male zebra finches (Blount *et al*., 2003; McGraw & Ardia, 2003). After moult was completed in the autumn experiment, we again scored plumage hue and saturation (as above) to determine the effect of dietary carotenoid supplementation on colour development.

Laboratory assays of carotenoids and immunity

Plasma carotenoid content

Solvent-extraction and high-performance liquid chromatography (HPLC) analytical methods follow those in McGraw *et al*. (2006a). Carotenoids were sequentially extracted from 10 μL thawed plasma with 100 μL each of ethanol and 1 : 1 hexane : *tert*-butyl methyl ether. We centrifuged the solution at 8000 r.p.m. for 4 min, transferred the supernatant to a fresh tube, evaporated it to dryness under a stream of nitrogen, and resuspended the pigment residue in 200 μL methanol. Pigment extracts were injected into a Waters Alliance 2695 HPLC system fitted with a Waters YMC Carotenoid 5.0 μm column (4.6 × 250 mm) and a built-in column heater set at 30 °C. We used a three-step gradient solvent system to analyze both polar and nonpolar carotenoids in a single run, at a constant flow rate of 1.2 mL min^{-1}: first, isocratic elution with 42 : 42 : 16 (v/v/v) methanol:acetonitrile:dichloromethane for 11 min, followed by a linear gradient up to 42 : 23 : 35 (v/v/v) methanol : acetonitrile : dichloromethane for 20 min, held isocratically at this condition until 28 min, and finishing with a return to the initial isocratic condition from 28–40 min. Data were collected from 250–600 nm using a Waters 2996 photodiode array detector. We identified pigments by comparing their respective retention times and absorbance maxima (λ_{max}) to those of purified, reference carotenoids. Circulating carotenoid concentrations were determined by comparison with external standard curves for each pigment. We detected lutein, zeaxanthin, and β-cryptoxanthin as major plasma carotenoids, which is typical for this species (McGraw *et al*., 2006a).

Bacterial-killing efficiency

To 40 μL fresh whole blood that we collected within 10 min of entering the birds' room and that we returned to the laboratory for analysis within 30 min of collection (*sensu* Matson *et al*., 2006), we added 200 *E. coli* (E^{power} microorganisms ATCC #8739, 10^7, MicroBioLogics) suspended in medium (CO_2 independent medium + 4 mM glutamine + 5% heat-inactivated fetal calf serum) to give a final dilution of 1 : 10. The mixture was incubated at 37 °C for 30 min, at which point we transferred 75-μL aliquots of each sample to two 4% tryptic soy agar plates, dispersed the solution homogenously across the plate with a sterile glass spreader, and incubated the plate overnight at 37 °C. We returned the next morning to count the number of bacterial colonies per plate and determined killing efficiency (% colonies killed) relative to control plates prepared only with medium and *E. coli*. Killing efficiency was highly repeatable for our duplicate samples (all $R_i > 0.95$; *sensu* Lessells & Boag, 1987) from all four sampling periods (pre- and post-treatment in winter and fall experiments), so we used averages in statistical analyses.

Response to PHA injection

We first measured the thickness of the left patagium three times with a pressure-sensitive micrometer (Mitutoya Inc.) to the nearest 0.001 mm and then injected 50 μL of phosphate-buffered saline containing 0.25 mg PHA (Sigma Chemical Co.) into the left patagium of each bird (*sensu* Deviche & Cortez, 2005). We returned 24 h later to measure patagium thickness in the same fashion. Repeatability of patagium swelling (calculated as post-pre patagium thickness) was high both before and after injection in both experiments (all $R_i > 0.8$), so again we used averages in statistical analyses.

Statistical analysis

For all variables, we tested the assumptions of parametric statistics (normality and homoscedasticity) and rank transformed them (Conover & Iman, 1981) when assumptions were violated. We ran repeated-measures analyses of variance (rmANOVAs) using sex, dietary carotenoid treatment, time, and their interactions as predictors of carotenoid accumulation and bacterial killing efficiency. We used two-way ANOVAs to test for diet, sex, and diet × sex differences in our single-time-point variables: plumage coloration (pre-experiment in winter study, post-experiment in autumn molt study) and post-experiment wing-web swelling. Note that our sample

size declined from 32 in the winter 2004–05 experiment to 26 in the autumn 2005 experiment (six control females, five supplemented females, seven control males, eight supplemented males) because of unexpected disease (avian pox) and mortality that occurred in the intervening spring and summer. No other birds in the present study showed signs of pox infection. Data are presented as the mean ± SE in all cases, and α-level was set to 0.05 throughout.

RESULTS

Carotenoid supplementation and immunity during winter

Before the experiment, treatment groups did not differ in plumage hue ($F_{1,28} = 0.09$, $P = 0.77$) or saturation ($F_{1,28} = 0.02$, $P = 0.89$), nor were there any significant sex × diet interactions (both $P > 0.2$). We did, however, find the expected sex differences in plumage hue ($F_{1,28} = 73.4$, $P < 0.0001$) and saturation ($F_{1,28} = 92.9$, $P < 0.0001$), with male plumage being redder and more saturated than that of females.

We confirmed that our dietary carotenoid manipulation created an experimental difference in carotenoids accumulated in the body, as demonstrated by the significantly elevated levels of all carotenoids in the plasma of supplemented birds compared to controls, although only after the experiment (i.e. the significant diet × time factors in Fig. 1, Table 1). Levels of all three carotenoids were highly positively correlated within a bird, even when we broke down analyses by sex and treatment group (all $r > 0.84$, all $N = 8$, all $P < 0.002$). Over the course of the experiment, supplemented males and females increased in total plasma carotenoid concentration by 63 ± 24% and 132 ± 24%, respectively, whereas plasma carotenoid levels slightly decreased in control males and females during this experiment (–1 ± 7% and –13 ± 8%, respectively). Plasma carotenoid levels for all but two supplemented females (that circulated 65 μg mL^{-1}) fell within the natural range of variation that we have previously detected at this time of year in our population of wild house finches (2–50 μg mL^{-1}), and mean levels for control birds (14.8 μg mL^{-1}) closely approximated the average among free-ranging adults from November to January (14.2 μg mL^{-1}, $N = 115$; K. J. McGraw, unpubl. data). There were no sex differences in plasma carotenoid levels in the experiment, nor diet × sex interactions, although we did find significant diet × sex × time interactions for all three carotenoids, with plasma carotenoid concentrations increasing more over time in supplemented females than in supplemented males (Fig. 1, Table 1).

We found no effect of dietary carotenoid treatment ($F_{1,28} = 0.04$, $P = 0.85$), sex ($F_{1,28} = 0.21$, $P = 0.65$), or the diet × sex interaction ($F_{1,28} = 0.05$, $P = 0.82$) on wing-web swelling after the experiment (Fig. 2A). Bacterial killing efficiency also was unaffected by diet, sex, time, or any interaction term (Fig. 2B, Table 1). Killing efficiency changed only slightly, decreasing by 3%, over the course of the experiment (pre-treatment = 90.8 ± 3.4%; post-treatment = 87.7 ± 3.5%, for all birds combined). Even if we excluded the two females that circulated potentially unnaturally high levels of carotenoids through blood, there was still no health-enhancing effect of carotenoids for either immune measure (all $P > 0.3$).

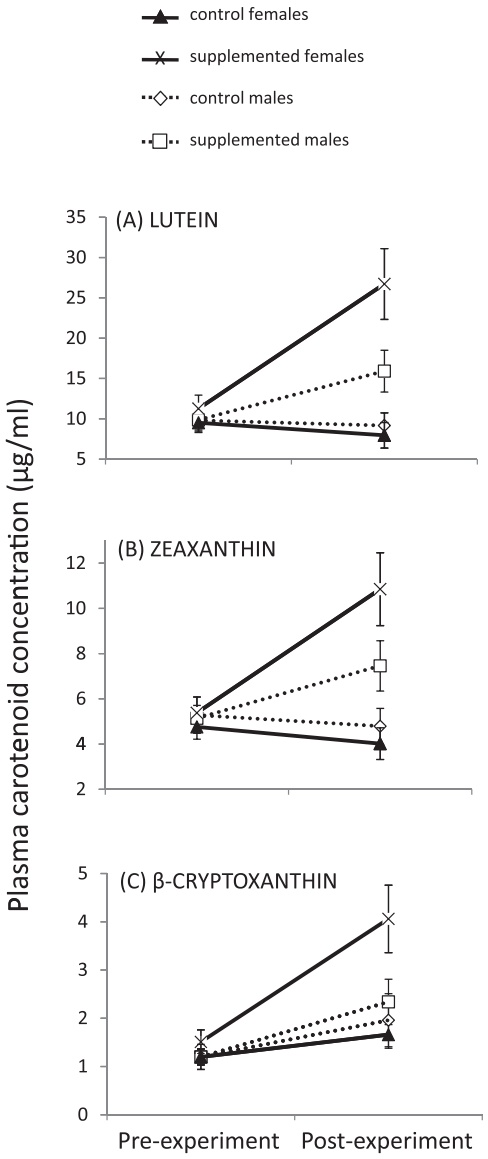

Figure 1. Effect of dietary carotenoid supplementation on accumulation of three carotenoid types (A, lutein; B, zeaxanthin; C, β-cryptoxanthin) in plasma of captive house finches during winter.

Table 1. Results of repeated-measures analyses of variance testing the effects of diet treatment, sex, time, and all possible interactions on plasma carotenoid concentrations, body mass, and bacterial killing efficiency in captive house finches from the winter experiment

Variable	Diet	Sex	Time	Diet × Sex	Diet × Time	Sex × Time	Diet × Sex × Time
Plasma lutein concentration ($\mu g\ mL^{-1}$)	$\mathbf{F_{1,28} = 12.10}$ $\mathbf{P = 0.002}$	$F_{1,28} = 1.85$ $P = 0.18$	$\mathbf{F_{1,28} = 23.79}$ $\mathbf{P < 0.0001}$	$F_{1,28} = 3.04$ $P = 0.09$	$\mathbf{F_{1,28} = 35.69}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{1,28} = 4.57}$ $\mathbf{P = 0.04}$	$\mathbf{F_{1,28} = 6.79}$ $\mathbf{P = 0.01}$
Plasma zeaxanthin concentration ($\mu g\ mL^{-1}$)	$\mathbf{F_{1,28} = 8.93}$ $\mathbf{P = 0.006}$	$F_{1,28} = 0.50$ $P = 0.49$	$\mathbf{F_{1,28} = 18.68}$ $\mathbf{P = 0.0002}$	$F_{1,28} = 2.15$ $P = 0.15$	$\mathbf{F_{1,28} = 35.23}$ $\mathbf{P < 0.0001}$	$F_{1,28} = 3.68$ $P = 0.06$	$\mathbf{F_{1,28} = 5.06}$ $\mathbf{P = 0.03}$
Plasma β-cryptoxanthin concentration ($\mu g\ mL^{-1}$)	$\mathbf{F_{1,28} = 5.00}$ $\mathbf{P = 0.03}$	$F_{1,28} = 1.56$ $P = 0.22$	$\mathbf{F_{1,28} = 40.31}$ $\mathbf{P < 0.0001}$	$F_{1,28} = 2.84$ $P = 0.10$	$\mathbf{F_{1,28} = 10.16}$ $\mathbf{P = 0.003}$	$F_{1,28} = 2.04$ $P = 0.16$	$\mathbf{F_{1,28} = 4.84}$ $\mathbf{P = 0.04}$
Body mass (g)	$F_{1,28} = 0.01$ $P = 0.91$	$F_{1,28} = 0.05$ $P = 0.83$	$\mathbf{F_{1,28} = 11.41}$ $\mathbf{P = 0.002}$	$F_{1,28} = 0.10$ $P = 0.75$	$F_{1,28} = 1.13$ $P = 0.30$	$F_{1,28} = 2.76$ $P = 0.11$	$F_{1,28} = 0.02$ $P = 0.90$
Bacterial killing efficiency (%)	$F_{1,28} = 0.89$ $P = 0.35$	$F_{1,28} = 0.25$ $P = 0.62$	$F_{1,28} = 0.36$ $P = 0.55$	$F_{1,28} = 0.04$ $P = 0.85$	$F_{1,28} = 0.87$ $P = 0.36$	$F_{1,28} = 0.17$ $P = 0.69$	$F_{1,28} = 0.12$ $P = 0.74$

Bold terms indicate statistically significant effects.

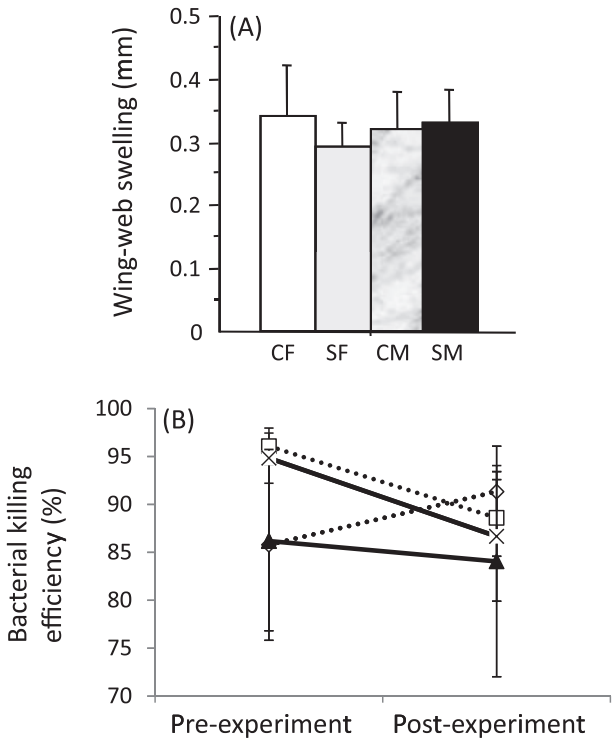

Figure 2. Effect of dietary carotenoid supplementation on two aspects of immunocompetence, namely (A) 24-h swelling of wing web tissue in response to injection with phytohemagglutinin after the experiment and (B) effectiveness of immune-system constituents in blood in killing introduced bacteria, in captive house finches during winter. CF, control females; SF, supplemented females; CM, control males, and SM, supplemented males. For symbol and line codes, see Fig. 1.

There also were no effects of carotenoid treatment, sex, or any interaction on body mass, although birds did gain weight over the course of the experiment (Table 1).

We had high statistical power (= 0.72–0.83, at $\alpha = 0.05$; Cohen, 1988) to detect the previously reported, strong effects of carotenoids on immunity in other studies on colourful adult birds ($r = 0.65$–0.75; Blount *et al*., 2003; McGraw & Ardia, 2003), which used comparable sample sizes ($N = 10$ per treatment group), as well as to detect those effects uncovered in the present study in autumn (see below).

Carotenoid supplementation, coloration, and immunity during moult

Our 8-week carotenoid supplementation experiment during autumn was again effective in generating differences in circulating carotenoids between treatment groups, although it had different and more complex effects on pigment accumulation than during the previous winter experiment. Plasma levels of the most

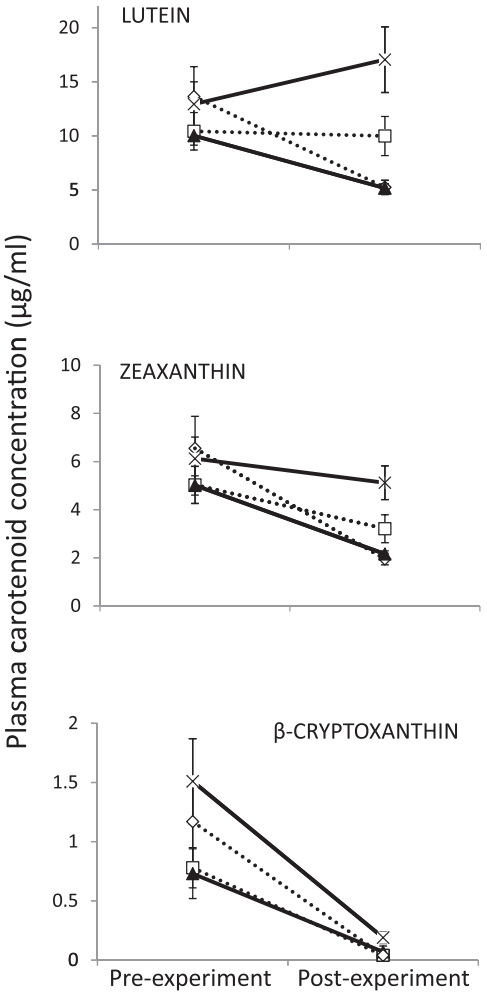

Figure 3. Effect of carotenoid supplementation on plasma carotenoid accumulation in captive house finches during autumn moult. For symbol and line codes, see Fig. 1.

concentrated carotenoid we manipulated (i.e. lutein) were significantly elevated in supplemented birds compared to control birds after the experiment, although the effect was stronger in females than in males (Fig. 3A, Table 2). Indeed, control birds of both sexes significantly declined (paired *t*-tests, both $P < 0.005$) in circulating carotenoid concentration during molt, although levels in supplemented females increased ($P = 0.03$) and those in males remained unchanged ($P = 0.68$; Fig. 3A). By contrast, circulating concentrations of the other, minor carotenoid we supplemented (i.e. zeaxanthin) decreased significantly in all birds over the experiment (Fig. 3B, Table 2; also $P < 0.01$ for all paired *t*-tests), although the significant diet × time interaction (Table 2) indicates that levels in supplemented birds decreased less than did those in control birds (Fig. 3B).

As in the winter experiment, plasma lutein and zeaxanthin concentrations were tightly intercorre-

Table 2. Results of repeated-measures analyses of variance testing the effects of dietary carotenoid treatment, sex, time, and all possible interactions on plasma carotenoid concentrations, body mass, and bacterial-killing efficiency in captive house finches from the autumn moult experiment

Variable	Diet	Sex	Time	Diet × Sex	Diet × Time	Sex × Time	Diet × Sex × Time
Plasma lutein concentration ($\mu g\ mL^{-1}$)	$\mathbf{F_{1,22} = 5.66}$ $\mathbf{P = 0.03}$	$F_{1,22} = 0.73$ $P = 0.40$	$\mathbf{F_{1,22} = 9.20}$ $\mathbf{P = 0.006}$	$F_{1,22} = 3.73$ $P = 0.07$	$\mathbf{F_{1,22} = 28.73}$ $\mathbf{P < 0.001}$	$\mathbf{F_{1,22} = 6.64}$ $\mathbf{P = 0.02}$	$F_{1,22} = 0.10$ $P = 0.75$
Plasma zeaxanthin concentration ($\mu g\ mL^{-1}$)	$F_{1,22} = 1.99$ $P = 0.17$	$F_{1,22} = 0.39$ $P = 0.54$	$\mathbf{F_{1,22} = 54.86}$ $\mathbf{P < 0.0001}$	$F_{1,22} = 2.59$ $P = 0.12$	$\mathbf{F_{1,22} = 11.15}$ $\mathbf{P = 0.003}$	$F_{1,22} = 3.41$ $P = 0.08$	$F_{1,22} = 0.46$ $P = 0.50$
Plasma β-cryptoxanthin concentration ($\mu g\ mL^{-1}$)	$F_{1,22} = 0.61$ $P = 0.44$	$F_{1,22} = 0.52$ $P = 0.48$	$\mathbf{F_{1,22} = 60.96}$ $\mathbf{P < 0.0001}$	$F_{1,22} = 4.28$ $P = 0.0504$	$F_{1,22} = 0.29$ $P = 0.59$	$F_{1,22} = 0.04$ $P = 0.84$	$\mathbf{F_{1,22} = 4.47}$ $\mathbf{P = 0.046}$
Body mass (g)	$F_{1,22} = 0.02$ $P = 0.89$	$F_{1,22} = 0.01$ $P = 0.94$	$F_{1,22} = 0.12$ $P = 0.73$	$F_{1,22} = 1.16$ $P = 0.29$	$F_{1,22} = 2.52$ $P = 0.13$	$\mathbf{F_{1,22} = 5.33}$ $\mathbf{P = 0.03}$	$\mathbf{F_{1,22} = 4.56}$ $\mathbf{P = 0.044}$
Bacterial killing efficiency (%)	$F_{1,22} = 3.98$ $P = 0.059$	$F_{1,22} = 0.27$ $P = 0.61$	$\mathbf{F_{1,22} = 70.35}$ $\mathbf{P < 0.0001}$	$F_{1,22} = 0.11$ $P = 0.74$	$\mathbf{F_{1,22} = 4.51}$ $\mathbf{P = 0.045}$	$F_{1,22} = 0.22$ $P = 0.65$	$F_{1,22} = 0.15$ $P = 0.71$

Bold terms indicate statistically significant effects.

lated within individual birds, even when broken down by sex and treatment group (all $r > 0.94$, all $P < 0.002$), although the concentrations of the carotenoid we did not supplement (i.e. β-cryptoxanthin) were not always significant correlated with lutein or zeaxanthin titers (r-values from 0.33–0.82, P-values from 0.49–0.01 for the different treatment groups). β-cryptoxanthin concentrations decreased to zero (in 11 of 26 birds) or nearly so during the study (Fig. 3C, Table 2). However, plasma carotenoid titres for no individuals in this experiment fell outside of the natural range of variation observed in wild moulting house finches in the present study population (2–34 μg mL^{-1}; McGraw *et al*., 2006a); levels of total circulating carotenoids in one supplemented female reached the maximum (34 μg mL^{-1}), although concentrations in all others were < 28 μg mL^{-1}. Moreover, levels of β-cryptoxanthin were not pharmacologically low because 27 of 57 birds in our previous study of plasma carotenoids in wild moulting house finches circulated < 0.4 μg mL^{-1} β-cryptoxanthin (McGraw *et al*., 2006a). Presumably, carotenoid levels generally declined in moulting birds as a result of depressed health (see below) and/or active carotenoid allocation to plumage, except in females (who deposit fewer carotenoids into plumage than do males) that received carotenoid supplements. The fact that there were no group differences in levels of any carotenoid type before the experiment indicates that there were no carry-over effects of the prior (winter) supplementation on carotenoid accumulation in the present study.

We found no effect of carotenoid supplementation on the hue of newly moulted carotenoid-based plumage ($F_{1,24} = 0.64$, $P = 0.43$), although the sexes again differed significantly ($F_{1,24} = 8.98$, $P = 0.007$; sex × treatment interaction: $F_{1,24} = 3.19$, $P = 0.09$). The lack of an effect on hue was not surprising because all birds were fed a diet with very low β-cryptoxanthin levels and thus developed drab yellow plumage. However, we did find a significant treatment effect on plumage saturation ($F_{1,24} = 5.58$, $P = 0.028$), with carotenoid-supplemented birds developing more saturated (6% more in males, 26% in females) yellow plumage than controls; as expected, males also developed more saturated plumage than females ($F_{1,24} = 68.0$, $P < 0.0001$; sex × treatment interaction: $F_{1,24} = 0.14$, $P = 0.71$). Body mass did not differ significantly as a function of diet, sex, or time, although there were significant sex × time and diet × sex × time interactions, such that supplemented females differed from the rest of the groups by losing, as opposed to gaining, mass (Fig. 4A, Table 2).

Finches showed exceptionally high bacterial-killing efficiency just prior to moult (99.7 ± 0.09%), indicating that birds were still in excellent health after spending more than 8 months in captivity. Moreover, we found

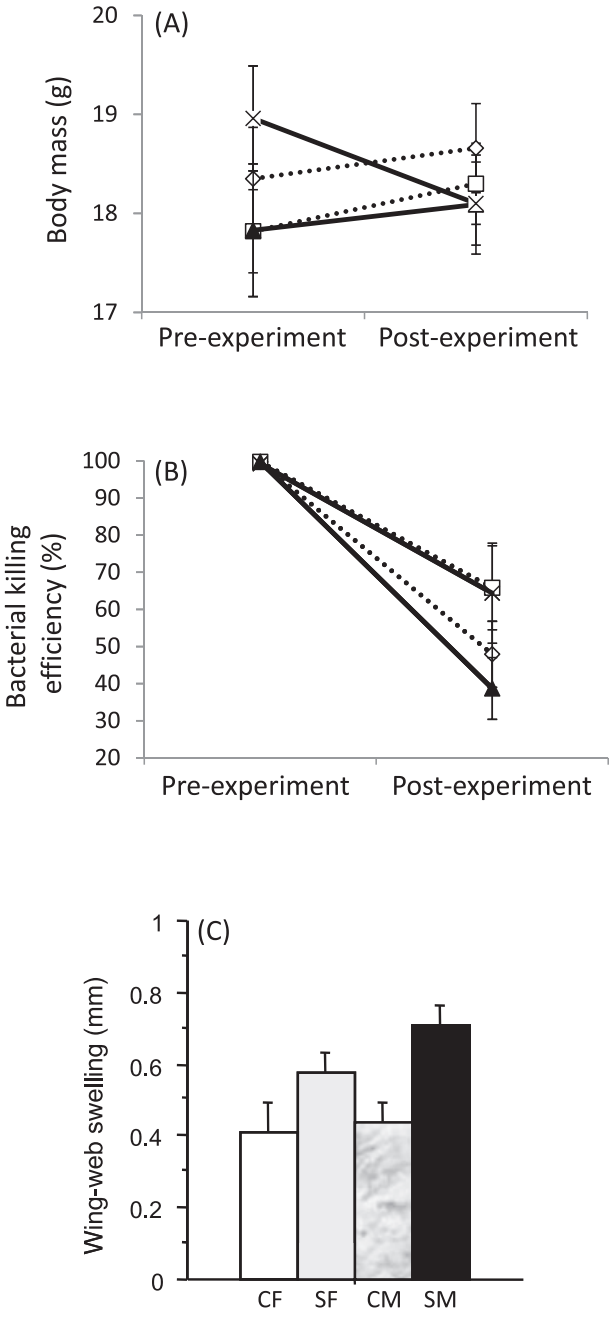

Figure 4. Effect of carotenoid supplementation on (A) body mass, (B) bacterial killing efficiency, and (C) wing-web swelling response to phytohemagglutinin in captive house finches during autumn moult. For symbol and line codes, see Fig. 1.

no pre-experiment differences among groups in bacterial-killing efficiency of blood (Fig. 4C), again pointing to no carry-over effects from the winter study. However, unlike the results of the winter experiment, we found that carotenoid supplementation during moult enhanced both of our measures of

immune performance in carotenoid-supplemented birds relative to controls (Fig. 4). First, there was a significant effect of the diet treatment × time interaction on bacterial-killing efficiency (Table 2). Although bacterial-killing efficiency declined substantially during moult (down to 54.5 ± 5.5%; time effect in Table 2), on average, supplemented females and males killed 26% and 18%, respectively, more bacterial colonies after the experiment than did control females and males (Fig. 4B). Second, diet treatment had a strong effect on post-experiment wing-web swelling ($F_{1,22} = 12.2$, $P = 0.002$). Wing web thickness increased 62% and 41% more in response to PHA after the experiment in treatment males and females, respectively, compared to control groups of each sex (Fig. 4C). There was no effect of sex ($F_{1,22} = 1.73$, $P = 0.20$) or the sex × treatment interaction ($F_{1,22} = 0.70$, $P = 0.41$) on wing-web swelling.

DISCUSSION

Carotenoids serve as valuable antioxidants or immunomodulators in plants (Bartley & Scolnik, 1995; Visoly-Fisher *et al*., 2006) and animals ranging from humans (Johnson, 2002) to commercially valuable crustacean, fish, bird, and other mammal species (Chew & Park, 2004; Babin, Biard & Moret, 2010). However, their role as limiting modulators of health in colourful birds has been debated (Shykoff & Widmer, 1996; Hill, 1999; Hartley & Kennedy, 2004; Costantini & Møller, 2008). We used a seasonal and experimental design to test the immunomodulatory properties of naturally occurring carotenoids in a wild songbird species that is classically known to use carotenoid-based plumage colour as an honest signal of nutrition and health (Hill, 2002). We found during the moult period that dietary carotenoid supplementation enhanced the performance of house finches in two constitutive innate immune challenges, although this same effect was absent in the same birds undergoing the same experimental manipulations during a nonmoulting (winter) period.

This demonstration of moult-specific carotenoid immunomodulation lends experimental support to Lozano's (1994) seminal 'trade-off' hypothesis for carotenoid-based colour signalling. The crux of his idea was that birds faced with devoting carotenoids to coloration are consequently limited in their ability to utilize carotenoids for becoming healthy, such that only those individuals with sufficiently high intake can satisfy the carotenoid requirements of both functions; in the present study, we show that immunocompetence in adult animals from a wild bird species is constrained by carotenoid accumulation, and specifically when carotenoids are being shunted to feathers for the development of sexual coloration.

Admittedly, our support for this hypothesis is still somewhat limited. We did not test for carotenoid immunomodulation at all other times of year in house finches, which would be necessary to conclude that the immune-enhancing benefits of high carotenoid supplies are reaped only during plumage moult. Moreover, our methods lacked the difficult but necessary molecule-specific tracking that would account for how pigments were specifically diverted to or traded-off for particular functions. For example, it is conceivable in the present study that carotenoids changed seasonally in their immunomodulatory properties not because carotenoids were lost directly to plumage in autumn, but simply because of alternate energetic stressors that occur during the moult period (e.g. oxidative stress, nutrient demands for feather replacement itself, increase in parasitism). Moreover, ideally we would conduct these manipulations and measurements in wild birds experiencing natural nutritional and energetic conditions. Nonetheless, among the avian studies to date that have confirmed carotenoid immunomodulation, the present study provides the best experimental support in a wild bird species that carotenoids are limiting agents when pigments can be used for both health and colour acquisition. Moreover, the health benefits of carotenoids have been understudied in female animals in this context (McGraw & Ardia, 2005) and, in the present study, we demonstrate boosts in immunocompetence to both sexes when provisioned with supplemental carotenoids during moult.

The molecular mechanisms by which carotenoids modulate immunity are poorly known in birds (McGraw & Klasing, 2006) and even in mammals and humans (Stahl, Ale-Agha & Polidori, 2002). Antioxidant protection of cells and tissues in the body (including metabolically active immune cells) is most often cited (Krinsky, 1989; Surai, 2002) but gene-regulatory, cell-signalling, and enzyme-inhibition mechanisms are receiving increased attention in the human-health literature (Stahl *et al*., 2002). For our two measures of innate immunity, both of which probe standing immune performance over a short time course, it is quite plausible that carotenoids primarily acted as free-radical scavengers, protecting the leukocytes and complement in circulation from oxidative damage and hence serving in an immunopermissive fashion (allowing the immune system to perform adequately when replete but inadequately when deplete). However, we are aware of no studies on carotenoids and health in wild animals that have simultaneously monitored these different immunomodulatory mechanisms for carotenoids. Clearly, in future work, it will be key to account for as many of these as possible, not to mention additional measures of adaptive immunity, as we continue to shape our

understanding of how globally limiting carotenoids are for immune system functioning (McGraw & Klasing, 2006).

In the present study, we manipulated yellow dietary xanthophylls, which are the two predominant carotenoids in finch diets (McGraw *et al*., 2006a) and which clearly can be used for both coloration and health-boosting in birds (Blount *et al*., 2003; McGraw & Ardia, 2003). However, the key molecular ingredient for developing sexually attractive red coloration in house finches is β-cryptoxanthin, a more environmentally scarce, less polar, orange dietary carotenoid that is the substrate for production of red ketocarotenoids that appear in plumage (Hill, 2000; Inouye *et al*., 2001; McGraw *et al*., 2006a); without this carotenoid, birds consistently grow drab yellow plumage (Hill, 2000, 2002). Because only circulating xanthophylls were affected by our dietary xanthophyll manipulation during the experiment (in autumn) in which we noted health benefits of supplementation, we can only make reference in the present study to the health benefits of added levels of these dietary carotenoids. β-cryptoxanthin was present at very low levels before moult and precipitously declined from circulation during the experiment, putatively as a result of its use as a precursor for plumage pigments. Because various carotenoid types (e.g. xanthophylls versus carotenes) can be differentially useful for health and coloration in birds (Fitze *et al*., 2007), it will be valuable in future studies to manipulate all possible dietary carotenoid types and combinations, to evaluate whether the important dietary precursor for red plumage development in house finches may in fact be limiting for health and coloration purposes.

Finally, although our findings agree with those of Aguilera & Amat (2007), the present study contrasts markedly with the other published experiment on carotenoids and immunity during moult in a wild passerine species for which a sexual signalling function is known for carotenoid coloration. Navara & Hill (2003) studied captive, moulting American goldfinches (a close relative of house finches) and failed to detect any effect of dietary xanthophyll (lutein + zeaxanthin) supplementation on multiple measures of disease and immunity, including one used in the present study (wing-web swelling). Methodological differences between the present study and theirs are many, including the duration of the carotenoid supplementation (3 months in their study), carotenoid dosages (approximately six-fold greater than that supplemented in the present study), housing conditions of the birds (they kept goldfinches in large groups in outdoor pens), and disease exposure (they inoculated birds with two naturally occurring parasites during the second half of moult), all of which may have differentially impacted carotenoid intake and allocation in the two species. However, there is also the possibility that avian species genuinely differ in their immune sensitivities to carotenoids, with some experiencing fewer limitations or needs for them. Ecological pressures such as disease (species, incidence, and virulence), moult (timing, rate, and extent of feather replacement and carotenoid coloration), and carotenoid intake certainly vary among species, as well as among individuals in certain species. Now that we have several comparative frameworks in place for understanding interspecific variation in carotenoid intake, circulation, and coloration (Tella *et al*., 2004; McGraw, 2005; Olson & Owens, 2005; Olson, 2006; Cohen *et al*., 2008), it is time to begin ecoimmunological investigations aiming to supplement such rich databases with information on disease and immunity.

In conclusion, we have uncovered carotenoid immunomodulation during plumage moult in a wild passerine bird with sexually selected carotenoid coloration. This demonstration in adult, colourful animals indicates that similar evidence for the health effects of carotenoids in birds is unlikely to be an artefact of domestication or a phenomenon specific to antioxidant-demanding neonates that lack sexual colours or to species with carotenoid-pigmented bare parts but, instead, the result of true carotenoid limitations for avian self-maintenance. The challenge now is to determine how pervasive such effects are, and thus how limited carotenoids are for health and pigmentation, in a variety of ornamentally coloured animal species.

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