Effect of Dietary Carotenoid Supplementation on Food Intake and Immune Function in a Songbird with no Carotenoid Coloration

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Received: May 24, 2006
Initial acceptance: May 28, 2006
Final acceptance: June 15, 2006 (S. A. Foster)

doi: 10.1111/j.1439-0310.2006.01280.x

Abstract

Studies of ornamental carotenoid coloration suggest that animals may have evolved specialized mechanisms for maximizing color expression and advertising their potential worth as a mate. For example, when given a choice of foods, many carotenoid-pigmented fishes and birds select the more colorful, presumably carotenoid-rich foods, and then accumulate these pigments at high levels in both the integument and systemically, in order to boost their immune system and hence directly advertise their health state with their colors. The majority of animals, however, do not exhibit sexually selected carotenoid coloration, which raises the question of whether they still optimize pigment intake and allocation in ways that boost endogenous accumulation and health. We tested the effect of carotenoid supplementation on food intake, carotenoid accumulation in blood, and both innate and adaptive immunity in male society finches (*Lonchura domestica*) – a non-carotenoid-colored estrildid finch relative of the zebra finch (*Taeniopygia guttata*; a species in which males do display sexually attractive and health-revealing carotenoid color). Males fed a carotenoid-rich diet for 2 wk did not consume more food than control males. Still, consumption of the carotenoid-rich diet for 2 wk significantly elevated circulating levels of carotenoids in blood in male society finches, yielding the potential for immune enhancement. In fact, carotenoid-enriched finches performed significantly better than control birds in our assay of constitutive innate immunity (bacterial-killing activity of whole blood), although not in our test of inducible adaptive immunity (response to a mitogenic challenge with phytohemagglutinin). These results suggest that affinities for carotenoid-rich foods may be particular to species with sexually selected carotenoid pigmentation, but that, as in humans and other mammals (e.g. mice, rats) without carotenoid color, the immune-boosting action of carotenoids is conserved regardless of the strength of sexual selection on pigment use.

Introduction

Carotenoid-based coloration is a common sexually selected trait in birds and fishes (Hill 2006). Their inexorable link to nutrition and their chemical tractability have made carotenoid colors fashionable models for investigating the costs and benefits of sexual features (McGraw 2006).

The last decade of work on the signal content of carotenoid colors has uncovered several apparently

specialized mechanisms that carotenoid-pigmented animals have evolved for achieving optimal carotenoid accumulation and hence sexually attractive coloration. Birds (e.g. house finches, *Carpodacus mexicanus*; Stockton-Shields 1997) and fishes (e.g. guppies, *Poecilia reticulata*, Rodd et al. 2002; sticklebacks, Smith et al. 2004) with sexually selected carotenoid coloration exhibit foraging preferences for the most colorful, red, presumably carotenoid-rich foods. There is also evidence from comparative studies that carotenoid-colored species of doves (Mahler et al. 2003) and passerines (Tella et al. 2004) consume more carotenoid-enriched diets than close relatives without such (or such intense) coloration. Independent of diet, colorful songbirds also seem to physiologically accumulate carotenoids at higher levels than other avian lineages where carotenoid coloration is less common (Tella et al. 2004; McGraw 2005). Finally, carotenoids are known to play important health-boosting roles in male birds and fishes with carotenoid coloration (e.g. zebra finches, *Taeniopygia guttata*, Blount et al. 2003; McGraw & Ardia 2003; guppies, Grether et al. 2004), which allows them to directly signal health state with their color intensity (Lozano 1994).

An important question that emerges from this work is whether or not these mechanisms have evolved uniquely in species with ornamental carotenoid pigmentation. There would seem to be several reasons besides coloration why animals should maximize carotenoid acquisition and accumulation from foods (e.g. eye protection, health enhancement). The few studies that have been carried out on food-color choice and immunomodulation in species that lack carotenoid coloration, or for which carotenoid coloration is not known to be a sexual signal, have, but not always, shown (a) dietary preferences for red food (e.g. McPherson 1988; Willson et al. 1990; Puckey et al. 1996; Gamberale-Stille & Tullberg 2001) and (b) carotenoid-derived health benefits (e.g. Haq et al. 1996; Saino et al. 2003; McGraw & Klasing in press). Ultimately, more rigorous studies are needed, such as those with phylogenetically paired designs (i.e. comparing effects in close relatives with and without carotenoid coloration), to better understand the ubiquity of carotenoid-accumulation strategies in animals.

In this study, we examined how carotenoid supplementation in the diet affected both food intake and health in male society finches (*Lonchura domestica*). Society finches are brown, black, and white in color and lack integumentary carotenoid pigmentation, but are closely related to a species with health-revealing and sexually attractive carotenoid coloration – the zebra finch (Burley & Coopersmith 1987; Blount et al. 2003). We experimentally supplemented the seed diet of society finches with xanthophyll carotenoids that commonly occur in granivorous diets and compared levels of food consumption and immune performance [as measured by two assays, one of which – the phytohemagglutinin (PHA) test – has been shown to respond to carotenoid elevation in zebra finches; Blount et al. 2003; McGraw & Ardia 2003] between control and carotenoid-provisioned individuals. Under the idea that birds have an affinity for accumulating more carotenoids whenever possible, we predicted that carotenoid-supplemented birds would consume more food (and thus carotenoids) than controls and would subsequently show superior immune performance; alternatively, it is possible that carotenoid-provisioned birds might consume less food, because compared with controls they need fewer grams of food to meet their daily carotenoid requirements.

Methods

Twenty unrelated male society finches were obtained for this study from a breeder when they were approx. 9 mo of age. Birds were housed individually in small cages in an IACUC-approved indoor room on the campus of Arizona State University (see McGraw 2005 for more details). Birds were fed a base diet of tap water and Avian Science Super Finch Bird Seed (Volkman Seed Company, Ceres, CA, USA) for approx. 6 mo prior to our study, at which time they were housed in two large flocks under non-breeding conditions in flight cages. This finch mix has a similar carotenoid content (approx. 8 μg/g) to that of the millet used in our feeding experiment (see below).

Feeding Study

We manipulated the carotenoid content of the diet by adding carotenoid beadlets (carotenoids embedded in starch; Roche Vitamins, Parsippany, NJ, USA) to the food source, which is a more realistic offering of the supplement than the typical addition to the drinking water that has been used in most previous avian studies (e.g. Hill 2000; Blount et al. 2003; McGraw & Ardia 2003). Because the basal finch seed diet contained a mix of seed types, we simplified presentation by using white millet only in our study. Lutein and zeaxanthin are the primary carotenoid components of finch seed (McGraw et al. 2001) and

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are circulated as the predominant pigments (along with their derivatives, anydrolutein and dehydrolutein) in the blood of society (and other estrildid) finches (McGraw et al. 2002; McGraw & Schuetz 2004; McGraw 2005); thus, our supplement contained a mixture of both lutein and zeaxanthin, in the ratio in which they occur in white millet (70:30; McGraw et al. 2001). Ten randomly selected birds were fed a white millet diet for 2 wk (from which they acquired approx. 7 μg/g dietary xanthophylls; McGraw et al. 2001), whereas the remaining 10 males were fed white millet + 20 μg/g lutein and 8 μg/g zeaxanthin, raising total dietary xanthophyll concentration to 35 μg/g. This level is well within the natural range of dietary carotenoid concentrations (0.5–80 μg/g) for wild granivorous songbirds (house finches; Hill et al. 2002). Seed was coated in 2% sunflower oil (which lacks carotenoids) to ensure that carotenoid beadlets would adhere to and were spread homogenously throughout the seeds; the same amount of oil was added to the plain millet diet of control birds as well. A purified or pelleted diet, in which carotenoids are naturally mixed (sensu Koutsos et al. 2003) could not be used with these adult birds because they, like their estrildid finch relatives, refuse to eat foods other than seeds they can husk.

Once this new diet was presented, food intake was quantified on seven randomly chosen days over the subsequent 2 wk. Pilot studies showed that society finches consume 1–4 g of seed in a 24-h period, so we offered each bird 10 g of fresh food per day to ensure that they were never food-limited. Seed was initially weighed, placed in primary deep dishes housed within secondary dishes that could collect any spilled seed, and then weighed again 24 h later to determine food consumption. We weighed each bird before the 2-wk supplementation period so that we could examine food intake on a total-amount as well as per-unit-body-mass basis. We found no difference in body mass between carotenoid-supplemented and control males prior to the study (ANOVA, $F_{1,18} = 1.49$, $p = 0.24$), indicating no expected treatment bias in food intake.

Immune Performance

We conducted our two assays of immunocompetence at the start (only for the bacterial-killing assay) and end of the 2-wk supplementation period. The first assay probed constitutive innate immunity by assessing the ability of immune proteins (e.g. complement) and cells (e.g. macrophages) in blood to kill bacteria (Tieleman et al. 2005). Within 10 min of entering the room (in order to prevent the stress response from impairing standing immunity), we drew 100 μl blood from each bird via the wing vein so that we could examine the bactericidal activity of the whole blood toward *Escherichia coli* (E^{power} microorganisms ATCC no. 8739, 10^7; MicroBioLogics, St. Cloud, MN, USA), as well as save plasma from a subsample of blood to determine pre- and post-treatment plasma-carotenoid concentration for control and carotenoid-supplemented groups using high-performance liquid chromatography (see McGraw 2005 for methods). This bacterial-killing assay requires fresh whole blood, so the samples were transported back to the laboratory for use within 40 min of collection. Approximately 200 *E. coli* cells were added to whole blood and medium (CO_2-independent medium + 4 mM glutamine + 5% heat-inactivated fetal calf serum) to give a final dilution of 1:10 and incubated at 37°C for 30 min. After incubation, 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 to count the number of bacterial colonies per plate and determined the average (for the two plates per bird) killing efficiency (% colonies killed) relative to control plates prepared only with medium and *E. coli*. Killing efficiency was highly repeatable for our duplicate samples ($R_i = 0.99$, $F_{39,40} = 215$, $p < 0.0001$), so we used averages in our statistical analyses.

The second assay is a commonly used mitogenic challenge with PHA that measures inducible adaptive immunity (Smits et al. 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). We first measured the thickness of the left patagium three times with a pressure-sensitive micrometer (Mitutoya Inc., Aurora, IL, USA) to the nearest 0.001 mm and then injected 50 μl of phosphate-buffered saline containing 0.25 mg PHA (Sigma Chemical Co., St. Louis, MO, USA) into the left patagium of each bird; PHA dose was adjusted per unit body mass from that used with zebra finches (McGraw & Ardia 2003). We returned 24 h later to measure patagium thickness in the same fashion. Repeatability of patagium swelling was high both before ($R_i = 0.79$, $F_{39,40} = 12.3$, $p < 0.0001$) and after injection ($R_i = 0.89$, $F_{39,40} = 26.5$, $p < 0.0001$), so again we used averages in statistical analyses.

Statistical Analyses

Food intake data (both raw and body mass-corrected values) were normally distributed, so we used repeated-measures ANOVA, followed by pairwise Scheffe's post hoc tests, to examine the effect of carotenoid supplementation on food consumption across the 2-wk study. We used multivariate analysis of variance (MANOVA) along with Scheffe's tests, to compare pre-treatment, post-treatment, and changes in levels of individual and total plasma carotenoids between carotenoid-supplemented and control males; we log-transformed post-supplementation concentrations of lutein and zeaxanthin to meet the assumption of normality. We performed one-way ANOVA on pre-injection, post-injection, and changes in patagium thickness in response to PHA, after log-transforming pre-injection thicknesses. Finally, we compared pre-supplementation, post-supplementation, and changes in bacterial killing efficiency between treatment groups using ANOVA and after log-transforming the latter two variables.

Results

We found no significant effect of carotenoid supplementation on food intake, when measured either as the number of grams of food per day (repeated-measures ANOVA, $F_{1,18} = 0.14$, $p = 0.72$; Fig. 1a) or as the number of grams of food per gram body mass per day ($F_{1,18} = 0.63$, $p = 0.44$; Fig. 1b). Food intake did increase over time in both treatment groups (g food/day: $F_{1,18} = 22.5$, $p < 0.0001$; g food/g body mass/day: $F_{1,18} = 20.0$, $p < 0.0001$; Fig. 1), which likely represents the gradual acclimatization of the birds to the novel food presentation.

Regardless of the lack of treatment difference in food intake, carotenoid-supplemented birds were still consuming four times more carotenoids than controls. Treatment groups did not differ in plasma-carotenoid profile prior to the feeding experiment (MANOVA, Wilks' lambda = 0.77, $F_{6,13} = 0.64$, $p = 0.70$), but our carotenoid supplementation created statistically significant differences in: (a) the concentration of carotenoids in blood after the 2-wk supplementation (Wilks' lambda = 0.28, $F_{6,13} = 5.64$, $p = 0.004$), and (b) the change in carotenoid levels over the course of the experiment (Wilks' lambda = 0.37, $F_{6,13} = 3.63$, $p = 0.02$; Fig. 2). Specifically, compared with control males, carotenoid-supplemented birds had higher post-treatment levels of and increased more in concentration for all but one (anhydrolutein) of the five carotenoids in serum (Fig. 2).

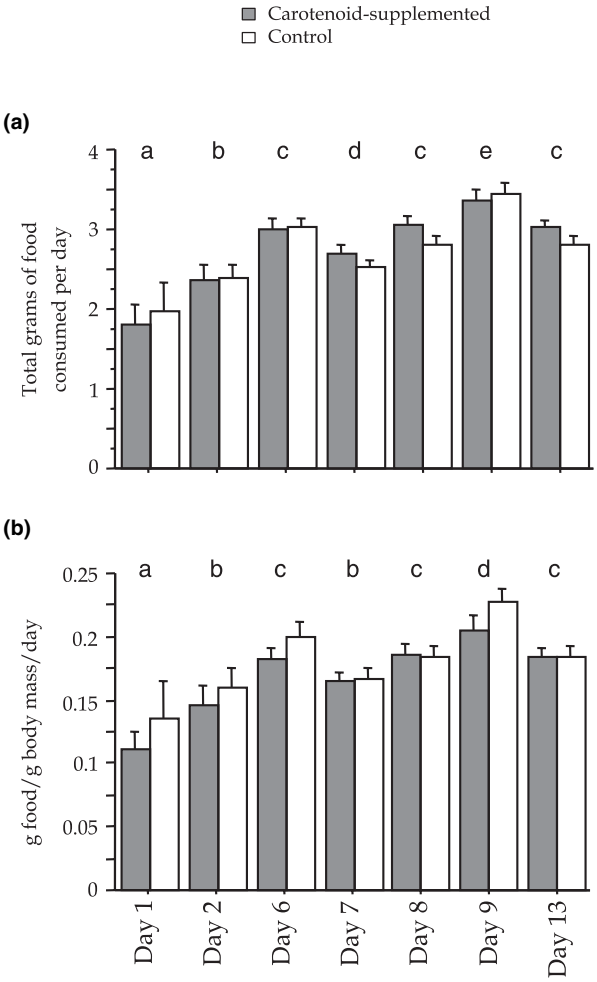

Fig. 1: Food consumption by individually caged male society finches fed either a seed diet or one supplemented with xanthophyll carotenoids for 2 wk. On seven randomly selected days during these 2 wk, food intake was calculated as: (a) total grams of food consumed per bird per day and (b) total grams of food consumed per gram of body mass per day. Pairwise differences in food consumption among the seven sampling days are denoted by letters atop the figure; shared letters denote non-significant differences ($p > 0.05$; Scheffe's tests)

Treatment groups did not differ significantly in *E. coli*-killing efficiency before ($F_{1,18} = 0.03$, $p = 0.87$) or after ($F_{1,18} = 0.76$, $p = 0.39$) the supplementation study, but carotenoid-provisioned males increased significantly more in killing efficiency (26%) than did controls (5%; $F_{1,18} = 4.5$, $p = 0.04$; Fig. 3a). In contrast, there was no significant effect of carotenoid treatment on the absolute post-injection intensity of ($F_{1,18} = 0.12$, $p = 0.74$) or 24 h change in ($F_{1,18} = 0.20$, $p = 0.66$) patagium swelling in response to PHA (Fig. 3b). However, PHA injection did have some biological effect on the birds, as body mass declined significantly during the 24 h period in

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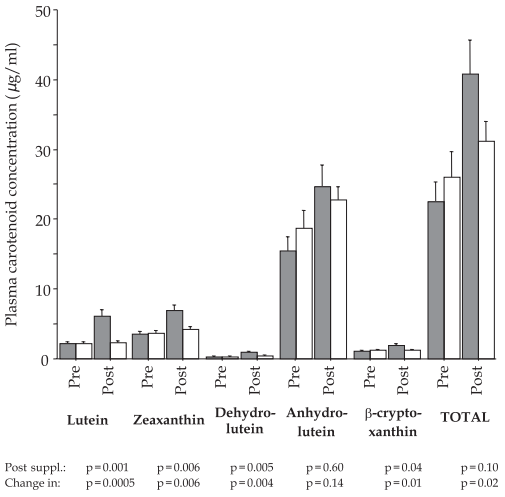

Fig. 2: Effect of 2-wk dietary carotenoid supplementation on levels of carotenoids in plasma, as determined by high-performance liquid chromatography (see McGraw 2005 for methods). Pre, pre-experimental levels; Post, post-experimental levels. Untransformed data (on post-supplementation lutein and zeaxanthin levels) are presented for clarity; p-values below the figure denote the significance of post hoc pairwise (Scheffe's test) comparisons for levels of each carotenoid between the two treatment groups; values are not shown for comparisons of pre-experiment carotenoid levels because the full-model MANOVA test for this time point was not statistically significant (see text)

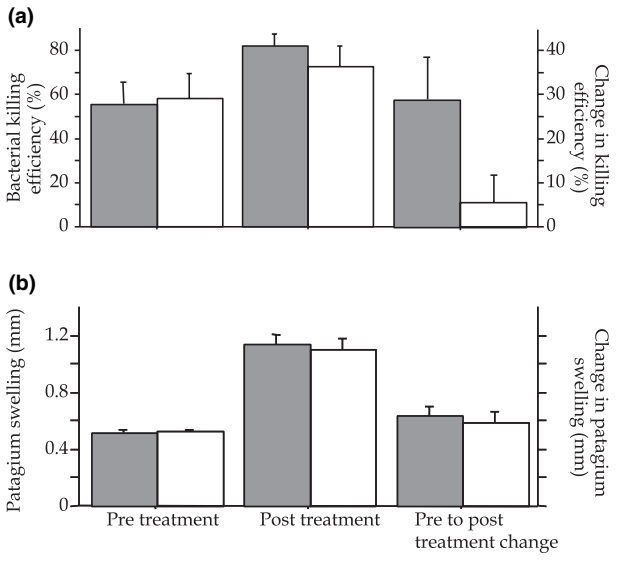

Fig. 3: Evidence for carotenoid immunomodulation in male society finches. Carotenoid supplementation increased (a) the strength of constitutive innate immunity, as measured by the bactericidal activity of whole blood samples toward *E. coli* (sensu Tieleman et al. 2005), but (b) did not affect the swelling response of individuals toward the injection of a novel mitogen (phytohemagglutinin) into the wing web

which they mounted a response to this mitogen (paired t-test, $t_{19} = -6.03$, $p < 0.0001$). There was no significant difference in body-mass change between carotenoid-supplemented and control males (unpaired t-test, $t_{19} = -1.4$, $p = 0.17$).

Discussion

Carotenoids and Diet

We found no influence of dietary carotenoid enrichment on rates of food consumption in captive male society finches. Most studies in fishes and birds that have investigated dietary preferences in relationship to carotenoid pigmentation have centered on food color, which has been represented by artificial objects (e.g. colored plastic strips or discs; Rodd et al. 2002; Smith et al. 2004) or manipulated artificially using paint or food coloring (Stockton-Shields 1997; Rodd et al. 2002). Our study is the first to test for carotenoid-related food consumption in songbirds. The failure of birds to increase food intake when it is carotenoid-rich has also been detected in domestic chickens (*Gallus gallus domesticus*, Haq et al. 1996; Koutsos et al. 2003) and Japanese quail (*Coturnix japonica*, Toyoda et al. 2002). Taken together, these findings support the important assumption of dietary carotenoid supplementation studies in birds that the observed effects (e.g. on coloration, health, reproduction) of dietary carotenoid enrichment are due to carotenoid molecules themselves, and not the other added nutrients that would be acquired if birds increased overall intake of food or water due to its experimentally high carotenoid content.

We provided a biologically relevant range of carotenoid supplies in the diet (7–35 μg/g), as our control and supplemental doses fell around the 25th and 90th percentiles, respectively, of median dietary carotenoid concentration in a wild granivore (house finches; Hill et al. 2002). We also are confident that carotenoid-supplemented birds did not simply preferentially pick out carotenoid beadlets from the outer oil coating on the seeds, as carotenoid levels in serum only increased two- to threefold in supplemented birds, compared with the fourfold higher levels available to them in food. Admittedly, this study did not test for a food-carotenoid or -color preference per se, but it does suggest, within a more natural range of variability in food conditions for this estrildid-finch species (i.e. as they are granivores and likely encounter low variability in seed color), that society finches modify their diet little in response to increased carotenoid supply. Studies of birds with

sexually selected carotenoid coloration should now also focus on the carotenoid content of foods in relation to food intake and selectivity.

Carotenoid-supplemented males did in fact accumulate higher levels of carotenoids in blood than did control males, which has been found in every avian carotenoid-supplementation study published, regardless of whether or not birds have carotenoid coloration (reviewed in McGraw 2005). However, society finches, like their zebra finch relatives, interestingly accumulate carotenoids at levels (ranging up to 67 μg/ml) approaching the highest ever reported from avian blood (Tella et al. 2004; McGraw 2005). This may be a product of domestication and strong artificial selection for the best cage birds (favoring carotenoid accumulation for antioxidant protection, egg yolk, etc.), but as they are a carotenoid-uncolored species, this suggests that bright coloration is not a prerequisite for high carotenoid accumulation abilities and is consistent with the notion that common ancestry is a strong predictor of carotenoid assimilation in birds (Tella et al. 2004; McGraw 2005).

Carotenoids and Health

Increased carotenoid accumulation in supplemented finches translated into bolstered constitutive innate immunity, as measured by stronger bactericidal activity of their blood towards *E. coli*. This is now the second study where bacterial killing efficiency was boosted by carotenoid provisioning in birds (also red junglefowl, *G. gallus*, McGraw & Klasing in press). This new assay (to studies of ecological immunity; Tieleman et al. 2005) may prove to be an ideal tool by which we can probe biologically relevant and interpretable, carotenoid-sensitive immune performance in other birds. Carotenoids may be acting either as immunopermissive antioxidants, offering protection to the standing cells and proteins in blood that make up the first line of defense against invading pathogens and allowing optimal function (Chew & Park 2004), or as gene regulators that induce the production of additional cells and proteins (Sharoni et al. 2002); understanding mechanisms of carotenoid immunomodulation, as has been done in humans, should become a priority in work on wild animals as well.

In contrast to the results of our bacterial-killing assay, responsiveness to PHA was not boosted in carotenoid-supplemented society finches. Variable effects of carotenoids on immunity have also been demonstrated in zebra finches (McGraw & Ardia 2005) and red junglefowl (McGraw & Klasing in press). We previously found that the PHA response of female zebra finches, unlike male zebra finches (Blount et al. 2003; McGraw & Ardia 2003) but like male society finches, was not sensitive to carotenoid status (McGraw & Ardia 2005). Perhaps the carotenoid demands of this line of adaptive, cell-mediated immune defense can be met by birds when there are fewer alternate uses for carotenoids (e.g. integumentary pigmentation), as in carotenoid-unpigmented society finches and scarcely carotenoid-pigmented female zebra finches, but become more difficult to satisfy when large amounts of carotenoids must be shunted elsewhere, as in heavily carotenoid-pigmented male zebra finches. These results underscore the inherent value in (a) converging on standard immune tests for interspecific, comparative purposes, as well as (b) selecting a broad battery of tests so that we can continue to understand the diverse effects that carotenoids can have on different arms of immunity in animals.

This is now the eighth avian species for which a health role of carotenoids has been implicated, either using direct evidence from a carotenoid supplementation experiment (domestic chicken, Haq et al. 1996; red junglefowl, McGraw & Klasing in press; barn swallow, *Hirundo rustica*, Saino et al. 2003; zebra finch, reviewed in McGraw & Ardia 2005; moorhen, *Gallinula chloropus*, Fenoglio et al. 2002) or indirectly via a health manipulation that depleted carotenoid stores (blackbird, *Turdus merula*, Faivre et al. 2003; mallard, *Anas platyrhynchos*, Peters et al. 2004). The majority of these species have carotenoid coloration, but some do not (red junglefowl, barn swallow; reviewed in McGraw 2005), indicating that the presence of carotenoid coloration is not a requirement for carotenoid immunomodulation in birds, as is the case in mammals (which all lack carotenoid coloration; Chew & Park 2004). Still, there are some species, even with ornamental carotenoid coloration, where carotenoids appear to play no immunoregulatory role (e.g. American goldfinch, *Carduelis tristis*, Navara & Hill 2003). We now need to supplement our database, rich in domesticated species without sexually selected carotenoid coloration, with more studies of wild species to better understand the true limitations and beneficial uses of carotenoids in nature.

Acknowledgements

The procedures reported here were approved by the Institutional Animal Care and Use Committee at

Arizona State University (protocol no. 05-764R). We thank S. Schenone and J. Badman for assistance with animal care, Roche Vitamins for donating carotenoid beadlets, M. Toomey, L. Taylor, and two anonymous referees for helpful comments on the manuscript, and the School of Life Sciences and College of Liberal Arts and Sciences at Arizona State University for funding.

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