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Immunoregulatory activity of different dietary carotenoids in male zebra finches

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Summary. Many animals use carotenoid pigments to color their integument and become sexually attractive. These colorants can also serve physiological functions, protecting cells and tissues from oxidative damage as well as stimulating the immune system. Because animals often acquire several different carotenoid pigments from their diet, there is the potential for different carotenoids to exhibit different free-radical-scavenging or immunoenhancing activity. We experimentally tested how two common dietary xanthophylls—lutein and zeaxanthin—may differentially affect the immune system in male zebra finches (*Taeniopygia guttata*). Male *T. guttata* derive their red sexual beak colorants from these two carotenoids, and prior studies with this species have shown that lutein and zeaxanthin together boost cell-mediated immunity. We experimentally elevated these two dietary carotenoids separately in two groups of non-breeding zebra finches, but found that lutein-supplemented and zeaxanthin-supplemented males mounted similar cell-mediated immune responses (to phytohaemagglutinin, or PHA). Although zeaxanthin is a more conjugated carotenoid than lutein and has the potential to be a more potent antioxidant, our study suggests that such a subtle structural difference between these two biochemicals does not differentially affect immune performance in this songbird.

Key words. Carotenoid pigments – cell-mediated immunity – lutein – phytohaemagglutinin – *Taeniopygia guttata* – zeaxanthin

Introduction

One means by which birds acquire their striking array of colors is through the incorporation of carotenoid pigments into feathers and bare parts (Stradi 1998). Carotenoids form a class of over 600 lipid-soluble organic compounds, many of which confer red, orange, and yellow pigmentation on birds and other colorful animals (Vershinin 1999). These extravagant feather colors are commonly used in intraspecific visual communication; male birds typically develop the most showy plumage and advertise their quality as a mate to females (Hill 1999).

Female birds can accrue several different benefits by mating with the most colorful, carotenoid-pigmented males (Hill 2002). Perhaps the most striking attribute of carotenoids as they relate to sexual signaling (aside from their light-absorbing capabilities) is their health function. Carotenoids are known to have potent antioxidant and immunomodulatory properties, scavenging free-radicals produced by cellular metabolism, protecting cells and tissues from oxidative damage, and directly stimulating the immune systems of animals (reviewed in Goodwin 1986; Bendich 1989). Thus, by obtaining large concentrations of carotenoids in the diet and displaying them in the integument, males can signal their nutritional and health state to prospective female mates (Lozano 1994).

There is now ample evidence in colorful birds that carotenoids are linked to both sexual coloration and immune function. For decades, correlational links between carotenoid-based integumentary coloration and nutritional/health state in birds have been elucidated (e.g. Hill & Montgomerie 1994; Saks *et al*. 2003). Two recent experimental studies in male zebra finches (*Taeniopygia guttata*), in which carotenoid status was manipulated, showed that supplemental pigment levels in the diet enhance carotenoid-derived red beak coloration as well as elevate cell-mediated immune performance (Blount *et al*. 2003; McGraw & Ardia 2003). In this line of research, however, few have considered the direct immunostimulatory properties of particular carotenoids. Animals like birds acquire several different xanthophylls and carotenes from the diet (Goodwin 1980; McGraw *et al*. 2001), all of which have different biochemical properties and may be differentially processed physiologically (Goodwin 1984). Thus, a detailed study of the immunoenhancing properties of different dietary carotenoids in birds is warranted.

In this study, we experimentally tested for differences in the immunostimulating action of two dietary xanthophylls—lutein and zeaxanthin—in male zebra finches. We chose the zebra finch as our study species since prior work demonstrated the direct, cell-mediated immunological boost provided by these two pigments when presented together as a diet supplement (Blount *et al*. 2003; McGraw & Ardia 2003). Lutein and zeaxanthin are the two most common yellow carotenoids found in plants and animals (Goodwin 1980,

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1984). Male zebra finches use these two xanthophylls that they obtain from their diet of weed and grass seeds (Zann 1996) as the major precursors for the production of red keto-carotenoids (McGraw *et al*. 2002) that enrich their sexually attractive beak (Burley & Coopersmith 1987). Here, we supplemented captive groups of male finches with either lutein or zeaxanthin and subsequently assayed their performance in a cell-mediated immune challenge (with the novel plant protein, phytohaemagglutinin, or PHA; Smits *et al*. 1999). Because the chromophore of zeaxanthin is more conjugated than that of lutein and thus has the potential to be a more potent antioxidant (Shimidzu *et al*. 1996; Mortensen & Skibsted 1997; Sujak *et al*. 1999), we predicted that zeaxanthin would be a superior immunoenhancer than lutein.

Methods

20 male zebra finches with wild-type plumage were housed in small wire cages (0.6 m long x 0.4 m wide x 0.4 m tall) on a 14:10 h light/dark cycle in an animal-approved indoor room on the campus of Cornell University. All birds were in non-breeding condition, housed without nest material or cups, and had similar breeding experience. We fed the finches an *ad libitum* diet of tap water, crushed oystershells, and Kaytee® Forti-Diet™ finch blend (Kaytee Products Inc., Chilton, WI) (see McGraw *et al*. 2002 for specific components of this seed mix). This diet is dominated by millet seeds and contains ca. 5 µg/g lutein and 2 µg/g zeaxanthin (McGraw *et al*. 2002). This seed diet generates carotenoid levels in birds that are comparable to those of other wild, colorful songbirds (details in McGraw & Ardia 2003).

In our previous study, we administered a supplemental dose of both lutein and zeaxanthin, as beadlets dissolved in the drinking water (Roche Vitamins Inc., Parsippany, NJ), to demonstrate that carotenoids boost the immune system of zebra finches (McGraw & Ardia 2003). In this experiment, we were interested in boosting levels of lutein in one experimental group (n = 10 males) and zeaxanthin levels in another (n = 10). Birds were assigned randomly to treatment groups and cages within each treatment were distributed randomly in our animal room. Since pilot tests showed that zebra finches consume approximately 2.5 g of food per day and drink 2.5 ml of water per day (McGraw & Ardia 2003), we were interested in doubling the daily intake of carotenoids in our groups, by adding 7 µg carotenoid/ml water to match the 7 µg/g they would obtain from food. Thus, to the drinking water of one of our groups, we added 7 µg/ml lutein, and to the other we added 7 µg/ml zeaxanthin. This diet treatment lasted one month, which was sufficient time in our previous study to elevate systemic carotenoid levels in males (McGraw & Ardia 2003).

After the one-month supplementation period, we drew blood from each bird to determine how the supplemental carotenoid dose affected blood-carotenoid levels. We also measured plasma-carotenoid status before the study to be sure that treatment groups did not differ initially. Following the methods of McGraw *et al*. (2002), we extracted carotenoids from plasma and used high-performance liquid-chromatography to quantify both the types and amounts in circulation. We thawed frozen plasma to room temperature and added 75 µl ethanol to 10 µl plasma. We vortexed the mixture and added 75 µl *tert*-butyl methyl ether. After vortexing again, we centrifuged the solution for 3 min in an Eppendorf centrifuge (model 5414). 150 µl of the supernatant was transferred to a new tube and evaporated to dryness under a stream of nitrogen. The remaining pigment residue was resuspended in 200 µl of HPLC mobile phase (methanol-acetonitrile-chloroform, 46:46:8, v/v/v) and vortexed prior to HPLC analysis. 50 µl of each sample was injected into a Waters™ 717plus Autosampler HPLC (Millipore Corp., Milford, MA) fitted with a Develosil RPAqueous RP-30 column (250 × 4.6 mm I.D.; Nomura Chemical Co., Ltd., Japan) and an Eppendorf TC-50 column heater (set at 32°C). An isocratic system (HP 1050 Series Isocratic Pump), using the aforementioned mobile phase for 25 min, was used for analysis at a constant flow rate of 1.2 ml/min. Using this procedure, 4 plasma pigments were previously identified in zebra finch plasma: three carotenoids present in the diet (lutein, zeaxanthin, and β-cryptoxanthin) and one metabolically derived pigment (2',3'-anhydrolutein) (McGraw *et al*. 2002, 2003). We determined the concentration of each of these carotenoids in plasma by comparing peak areas (integrated by Millenium™ software, version 2.1) to that of an internal standard (canthaxanthin, Roche Vitamins Inc., 1 µg/ml) that was added to each sample prior to pigment extraction.

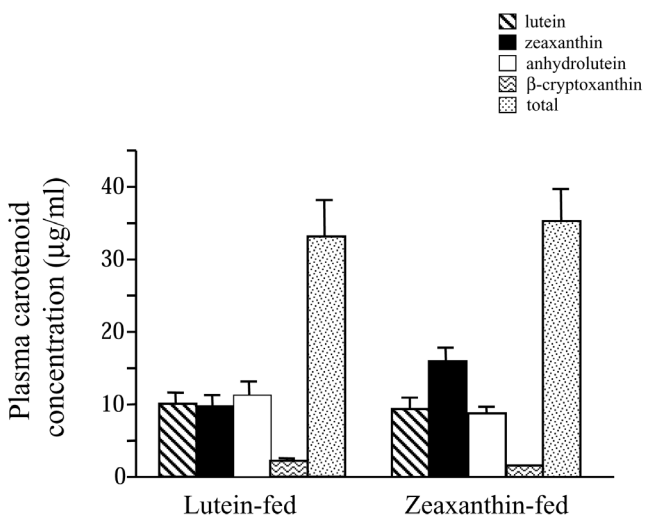

Fig. 1 Effect of dietary carotenoid supplementation on the plasma-carotenoid status of captive male zebra finches. Groups of ten birds were provisioned with either a dose of lutein or of zeaxanthin for four weeks (see Methods for details). Mean + SEM shown here and elsewhere

Also at the end of the supplementation period, we subjected males to a cell-mediated immune challenge. This 'PHA skin test' assays an individual's mitogenic, T-lymphocyte responsiveness to a foreign plant protein (Smits *et al*. 1999). This method has been used previously and effectively in assaying cell-mediated immunity in zebra finches (e.g. Ewenson *et al*. 2001) and in relation to carotenoid supplementation in humans (Kramer & Burri 1997) and mice (Jyonouchi *et al*. 1994). To conduct this immune test, we measured the right wing web of each bird 3 times with a digital micrometer (to the nearest 0.05 mm) to obtain an average pre-swelling measurement and then injected this area with 0.15 mg of PHA-P (Sigma Chemical Co., St. Louis, MO) in 30 ml phosphate buffered saline (PBS) (Hõrak *et al*. 1999). The birds were immediately placed back in their housing cages and we returned 24 hr later to measure the swollen area. We present results as the difference between mean post-injection swelling and mean pre-injection-swelling (*sensu* Smits *et al*. 1999). Within-individual repeatability of wing-web swelling measurements was moderately high (pre-injection: R_i = 0.55, F = 4.73, p = 0.04; post-injection: R_i = 0.62, F = 3.65, p = 0.05).

Data conformed to the assumptions of parametric statistics (e.g. normality, homoscedascity), so we used analyses-of-variance to compare blood-carotenoid levels and immune performance between treatment groups.

Results

Carotenoid levels

Prior to carotenoid supplementation, randomly assigned lutein-supplemented (LS) and zeaxanthin-supplemented

Table 1 Comparison of plasma-carotenoid signatures of lutein-supplemented (LS) and zeaxanthin-supplemented (ZS) male zebra finches prior to the experiment. Below, we report means ± s.e. and present carotenoid concentrations as μg pigment per ml plasma. We used ANOVA to examine group differences in all values

Measure	LS	ZS	$F_{1,18}$	p
[Lutein]	6.7 ± 1.5	8.0 ± 1.3	0.5	0.50
[Zeaxanthin]	6.7 ± 1.3	7.1 ± 0.7	0.1	0.80
[Anhydrolutein]	8.9 ± 1.4	11.0 ± 1.3	1.3	0.28
[β-cryptoxanthin]	2.1 ± 0.3	2.38 ± 0.3	0.6	0.44
[Total]	24.3 ± 4.2	28.5 ± 3.3	0.6	0.43

(ZS) males did not differ in any measure of plasma-carotenoid status (Table 1).

After the one-month supplementation period, LS and ZS males differed significantly in their blood-carotenoid profiles (Fig. 1). Not surprisingly, ZS males circulated significantly more zeaxanthin (63% more, on average) than did LS males ($F_{1,18}$ = 5.5, p = 0.03). LS males did not, however, circulate significantly more lutein than ZS finches ($F_{1,18}$ = 0.05, p = 0.82). This may be explained to some extent by the fact that lutein serves as the metabolic precursor to the formation of 2',3'-anhydrolutein and some β-cryptoxanthin in plasma (McGraw *et al*. 2002). Plasma from LS males did contain a significantly higher concentration (58% more, on average) of β-cryptoxanthin ($F_{1,18}$ = 4.8, p = 0.04) and a slightly higher (but not significantly so; 32% more, on average) concentration of anhydrolutein ($F_{1,18}$ = 2.0, p = 0.18) than that of ZS males. The two treatment groups did not differ in total plasma-carotenoid concentration ($F_{1,18}$ = 0.1, p = 0.75).

It is worth noting that circulating blood-carotenoid levels in these provisioned finches remained within the limits of non-provisioned finches in our previous studies (McGraw & Ardia 2003), indicating a reasonable, physiological dose in this study.

Cell-mediated immune response

Despite the fact that our two experimental groups had markedly different plasma-carotenoid profiles, generated by provisioining either supplemental lutein or zeaxanthin in the diet, we found that LS and ZS did not mount significantly different immune responses to PHA (Fig. 2; $F_{1,18}$ = 0.3, p = 0.59). Our sample sizes and supplemental carotenoid doses are identical to those in our previous study (McGraw & Ardia 2003), indicating that we had the statistical power to detect differences in cell-mediated immune function of a similar magnitude in this experiment. LS and ZS males in this study differed in cell-mediated immune response by only 3% (Fig. 2), whereas we previously found a 21% difference in wing-web-swelling between carotenoid-supplemented and control males (McGraw & Ardia 2003).

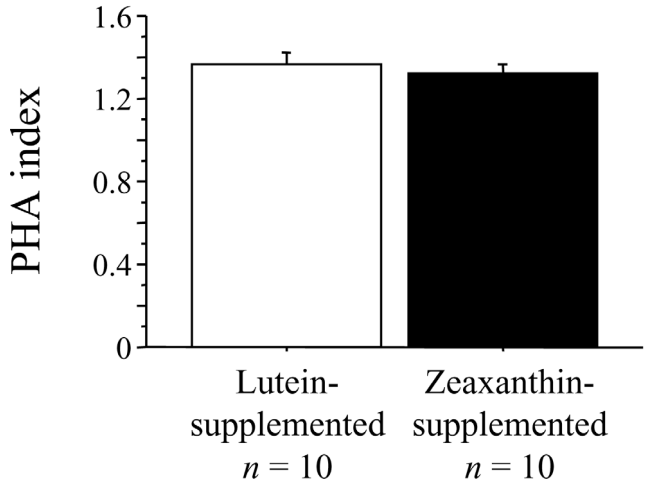

Fig. 2 Responses of lutein-supplemented (LS) and zeaxanthin-supplemented (ZS) male zebra finches to a cell-mediated immune challenge (with PHA)

Discussion

Carotenoids are touted as potent antioxidants and immunostimulants in humans (reviewed in Krinsky 2001; Hughes 2001) and several other animals (mammals: Chew *et al*. 1996; Zhao *et al*. 1998; Kim *et al*. 2000a, b; fishes: Tachibana *et al*. 1997; Amar *et al*. 2000, 2001). Mammals, including humans, are the best-studied taxa in this context, and in these groups the immunoenhancing capacities of particular carotenoids have been considered both observationally and experimentally (reviewed in Gerster 1993). Jyonouchi *et al*. (1996), for example, found that astaxanthin augments murine antibody production *in vitro*, whereas carotenoids like lutein and β-carotene do not exhibit such effects at an identical concentration. This finding is in agreement with the hypothesis that carotenoids with more conjugated double bonds are best able to quench free-radicals and thus serve as the most valuable antioxidants (Shimidzu *et al*. 1996).

Whether different carotenoids differentially stimulate the immune system has rarely been tested in animals like birds, however. It is only within the last few years that (a) the types of carotenoids present in birds have been identified unequivocally (Stradi 1998) and (b) carotenoids were reported to perform immunomodulatory roles in colorful birds (Blount *et al*. 2003). In this study, we provisioned captive zebra finches with a supplemental dose of either of two common dietary carotenoids (lutein and zeaxanthin) to examine whether one is a more potent immunoenhancer

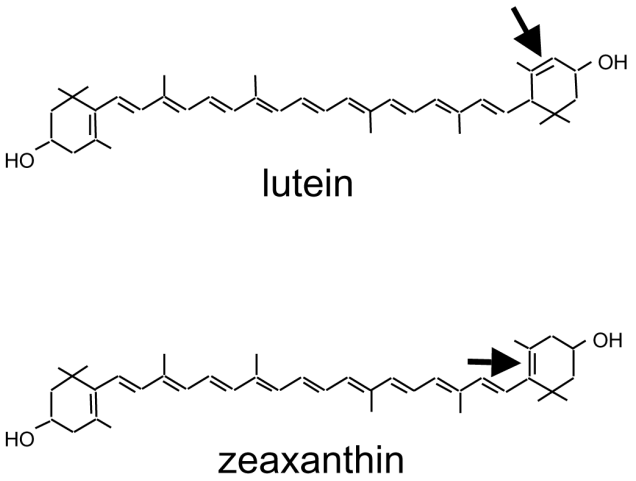

Fig. 3 Chemical structures of the two dietary carotenoids—lutein and zeaxanthin—manipulated in this study. Note the difference in double-bond conjugation between the two xanthophylls (denoted by arrows)

than another. We founded the prediction, based on the chemical structure of the two pigments (Fig. 3) and specifically the fact that zeaxanthin has a greater number of C=C double bonds in conjugation (11) than lutein (10), that zeaxanthin should be the stronger immunomodulator.

Although we demonstrated in a previous study that a combined supplement of lutein and zeaxanthin boosted cell-mediated immunity in male zebra finches (McGraw & Ardia 2003), here we show that identical concentrations of lutein and zeaxanthin do not differentially enhance this arm of the immune system. LS and ZS males had quite different pools of plasma carotenoids from which the immune system could draw to fight foreign pathogens, yet these two groups performed similarly in our immune challenge. We realize that our diet treatments were modest and that, in the end, we did not generate completely distinct treatment groups, where one group of males acquired/circulated lutein only and the other zeaxanthin only. However, we view this as a strength of our study, that we pushed plasma-carotenoid signatures to the extremes within the natural range of variation. Thus, to the extent to which zebra finches likely accumulate one pigment over another naturally from the diet, we can be confident that they have equal cell-mediated immunoregulatory activity.

Given their close structural similarity (differing only in the placement of a single double bond), it is not entirely surprising that zeaxanthin and lutein serve similar immunomodulating roles. Prior studies of the free-radical-quenching activity of various carotenoids have elucidated differences between pigments such as astaxanthin and lutein (Jyonouchi *et al*. 1996), which differ in conjugation by 3 double bonds. The carotenoids that zebra finches encounter in their granivorous diet or circulate through blood (also including anhydrolutein and β-cryptoxanthin; McGraw *et al*. 2002) share this same structural backbone (of 10-11 conjugated double-bonds), making it likely that any carotenoid-facilitated differences in immunostimulation can be attributed to overall pigment concentrations and not the presence of particular types. Perhaps a better model in which to test for differences in immunostimulation among dietary-carotenoid types would be a species with a more varied diet, such as gulls (e.g. *Larus fuscus*), where birds ingest carotenoids like astaxanthin and lutein that vary substantially in molecular structure (Blount *et al*. 2002).

Despite the fact that lutein and zeaxanthin had similar effects on the immune system in our study, we have found in previous experiments that they differ substantially in the extent to which they are preferentially accumulated in the body and contribute to sexual coloration in songbirds such as the zebra finch (McGraw *et al*., 2004). In fact, for a given concentration in the diet, zeaxanthin is more easily elevated in blood pools than lutein and subsequently generates a more colorful appearance in males (McGraw *et al.*, 2004). Thus, there may still be very important reasons why birds should actively seek out and enrich themselves with particular dietary carotenoids. Future studies like these that couple the physiological and morphological aspects of carotenoid pigments will continue to advance our understanding of how and why animals become colorful.

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