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Anhydrolutein in the zebra finch: a new, metabolically derived carotenoid in birds

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Abstract

Many birds acquire carotenoid pigments from the diet that they deposit into feathers and bare parts to develop extravagant sexual coloration. Although biologists have shown interest in both the mechanisms and function of these colorful displays, the carotenoids ingested and processed by these birds are poorly described. Here we document the carotenoid-pigment profile in the diet, blood and tissue of captive male and female zebra finches (*Taeniopygia guttata*). Dietary carotenoids including: lutein; zeaxanthin; and β-cryptoxanthin were also present in the plasma, liver, adipose tissue and egg-yolk. These were accompanied in the blood and tissues by a fourth pigment, 2′,3′-anhydrolutein, that was absent from the diet. To our knowledge, this is the first reported documentation of anhydrolutein in any avian species; among animals, it has been previously described only in human skin and serum and in fish liver. We also identified anhydrolutein in the plasma of two closely related estrildid finch species (*Estrilda astrild* and *Sporaeginthus subflavus*). Anhydrolutein was the major carotenoid found in zebra finch serum and liver, but did not exceed the concentration of lutein and zeaxanthin in adipose tissue or egg yolk. Whereas the percent composition of zeaxanthin and β-cryptoxanthin were similar between diet and plasma, lutein was comparatively less abundant in plasma than in the diet. Lutein also was proportionally deficient in plasma from birds that circulated a higher percentage of anhydrolutein. These results suggest that zebra finches metabolically derive anhydrolutein from dietary sources of lutein. The production site and physiological function of anhydrolutein have yet to be determined.

Keywords: Adipose tissue; Carotenoids; Diet; Egg yolk; Estrildid finches; Plasma carotenoids; Liver; *Taeniopygia guttata*

1. Introduction

Most of the flashy red, orange and yellow colors displayed by songbirds are the result of carotenoid pigments (Fox and Vevers, 1960; Stradi, 1998). Because vertebrates cannot synthesize carotenoids de novo, they must ultimately rely on dietary sources of pigment for integumentary coloration (Palmer, 1922; Völker, 1938; Goodwin, 1984). However, many birds are capable of metabolically converting ingested carotenoids into more oxidized and differently colored forms that are then incorporated into feathers and bare parts (Brush, 1990). At present, many aspects of these physiological transformations remain undescribed, including the sites of conversion, the responsible enzymes and the energetic requirements (McGraw and Hill, 2001).

To begin to understand the physiological processes that control carotenoid-based integumentary pigmentation, we must first document the carote-

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noid pigments present in the diet, plasma and tissues of colorful songbirds. This information will help determine if, where and how birds perform these metabolic conversions and to what extent they contribute to ornamental coloration. Although the identity of integumentary carotenoids has been reported recently for a number of avian taxa (e.g. Stradi, 1998; Negro and Garrido-Fernandez, 2000; Inouye et al., 2001; McGraw et al., 2001, 2002), the types and amounts of plasma and tissue carotenoids are unknown for these particular groups, and among birds have been determined only for poultry and certain gamebird and waterbird species (Fox and McBeth, 1970; Surai and Speake, 1998; Slifka et al., 1999; Royle et al., 1999, 2001), often with no knowledge of dietary carotenoid composition.

Here, we describe the carotenoid-pigment profile in the diet, plasma and tissues of captive male and female zebra finches (*Taeniopygia guttata*; Family Estrildidae). This songbird is native to Australia and consumes primarily grass and weed seeds throughout the year (Zann, 1996). Males display bright red carotenoid-based bill coloration, whereas females exhibit a less intensely pigmented orange beak (Burley and Coopersmith, 1987). We used HPLC techniques to determine the carotenoid content of a commercial finch food mix, and sampled blood from non-breeding birds and tissue from eggs and euthanized animals to identify the types and relative amounts of pigments present in plasma, liver, adipose tissue and egg yolk.

2. Methods

2.1. Animal use

We obtained animals for this study from an established colony of zebra finches at Cornell University (Adkins-Regan and Wade, 2001). Only non-breeding finches in wild-type plumage were used. We housed male–female pairs in small wire cages (0.6 m long$\times$0.4 m wide$\times$0.4 m tall) on a 14:10 h light/dark cycle in an animal-approved indoor room. All procedures followed during this study were approved by Cornell University's Institutional Animal Care and Use Committee (Protocol #99-89).

2.2. Diet samples

Birds were fed an ad libitum diet of water and Kaytee® Forti-Diet™ (Kaytee Products Inc., Chilton, WI). This mix contains 12 food components in the following proportions: white millet (19%); red millet (19%); golden yellow millet (19%); canary grass seed (19%); niger seed (7%); red fortified extruded supplement (4%); green fortified extruded supplement (4%); flax (2%); plain oat groats (2%); red-colored oat groats (2%); yellow-colored oat groats (2%); and green-colored oat groats (1%).

Finches consumed these different food types in direct proportion to the degree to which they were available in the diet (K. McGraw, unpublished data), so we quantified carotenoids from three separate 0.5-g portions of the full diet mix. We pulverized seeds to a fine powder with a mortar and pestle and extracted lipids three times with 2-ml tetrahydrofuran (McGraw et al. 2001). We centrifuged the suspension at 3000 rev./min for 3 min and evaporated the supernatant to dryness under a stream of nitrogen. The residue was redissolved in 1-ml HPLC mobile phase A (methanol-methylene chloride, 50:50, v/v, + 0.05% triethylamine) and 0.5 ml of mobile phase B (methanol-acetonitrile, 50:50, v/v, + 0.05% triethylamine) and stored at -20 °C overnight before centrifuging off the white precipitate and analyzing via HPLC (see below).

Because plant carotenoids often occur in esterified forms that are difficult to analyze chromatographically (Breithaupt and Bamedi, 2001), we also saponified an aliquot of each diet sample to cleave any fatty acids prior to HPLC analysis. We evaporated 100 ml of sample under nitrogen and added 1 ml of 5% potassium hydroxide in methanol. The tube was capped under argon and heated to 60 °C for 30 min. We cooled the sample to room temperature and added 1 ml of saturated sodium chloride, 4 ml distilled water and 8 ml hexane/tert-butyl methyl ether (1:1, v/v). The mixture was shaken vigorously for 2 min, centrifuged and the supernatant was removed and washed twice with water prior to evaporation and addition of the HPLC mobile phase.

2.3. Blood samples

We sampled blood from 12 pairs of male and female zebra finches on 29 December 1999. Whole blood (80–100 ml) was collected from the alar vein of each individual into heparinized microcapillary tubes. We centrifuged the tubes at 3000 rev./min for 10 min, removed the plasma with a syringe

and stored the samples in 1.5-ml Eppendorf tubes at −80 °C for later analysis. Carotenoids were extracted from thawed plasma by adding 200-ml ethanol to 25-ml plasma. We vortexed the mixture and added 100-ml tert-butyl methyl ether. After vortexing again, we centrifuged the solution for 3 min in an Eppendorf centrifuge (model 5414). The supernatant (200 ml) was transferred to a new tube and evaporated to dryness under nitrogen. The residue was dissolved in 200-ml mobile phase and vortexed prior to HPLC analysis. To determine whether plasma pigments may have occurred as esters or in free form, we also saponified plasma extracts following the de-esterification procedure outlined above for food carotenoids.

2.4. Tissue samples

Two male and 2 female zebra finches were briskly euthanized under a stream of carbon dioxide. We immediately dissected the birds and removed portions of the liver and adipose tissue. These tissues were stored at −80 °C prior to carotenoid extraction. We followed the previously described protocol for food carotenoids to isolate and prepare tissue pigments for HPLC analysis, except that final pigment residues were redissolved in 200 ml of a different HPLC mobile phase (methanol-acetonitrile-chloroform, 46:46:8, v/v/v, +0.05% triethylamine). Due to high lipid content, we saponified the adipose-tissue extract as above (also see Negro et al., 2001).

2.5. Egg samples

Starting in July 2000, we began breeding eight pairs of finches by providing caged birds with nesting material (shredded burlap) and nest cups (hanging Rubbermaid containers with an open top). As part of another study, we collected and weighed the 2nd- and 4th-laid eggs in these clutches and stored them at −80 °C. Frozen eggs were thawed, shelled and homogenized prior to carotenoid extraction. To each homogenate, we added 1-ml ethanol and 1-ml tert-butyl methyl ether prior to vortexing and collecting the supernatant for HPLC analysis.

2.6. HPLC and MS procedures

We analyzed carotenoids in the three finch food portions by injecting 10 ml of each sample into a Hitachi L-6200 HPLC (Hitachi Ltd., Tokyo, Japan) fitted with a Develosil RPAqueous RP-30 HPLC column (250×4.6 mm I.D.; Nomura Chemical Co., Ltd., Japan). We used separate isocratic systems to analyze carotenes (using HPLC phase A) and xanthophylls (using HPLC phase B) at constant flow rates of 1.2 ml min^{-1}. Carotenoids were detected at 450 nm using a Hitachi L-4250 UV/VIS detector, and peak areas were integrated with the HP 3390A integrator. We identified pigments present in the seed mix by comparing their retention times to those of authentic reference carotenoids provided by Roche Vitamins Inc. (lutein: 13.5 min; zeaxanthin: 15.6; β-cryptoxanthin: 27.5 min; β-carotene: 7.4 min). Percent composition was determined by comparing the ratios of peak areas generated by each pigment.

To determine the types and amounts of carotenoids in plasma, liver, adipose tissue and egg-yolks, we analyzed 50 ml of each sample with a Waters™ 717plus Autosampler HPLC (Millipore Corp., Milford, MA) fitted with the same RP-30 column. 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^{-1}. We again confirmed the identity of pigments by comparing retention times to authentic reference carotenoids (lutein: 6.0 min; zeaxanthin: 6.3 min; anhydrolutein: 13.0 min; β-cryptoxanthin: 19.3 min). Carotenoids were detected at λ_{max} for each pigment (lutein: 445 nm; zeaxanthin: 450 nm; anhydrolutein: 450 nm; β-cryptoxanthin: 450 nm) using a Waters™ 996 photodiode array detector (Waters Chromatography, Milford, MA).

Mass spectrometry data (see below) were obtained using an Esquire-LC ion trap (Bruker Daltonik GmBH, Bremen, Germany). Samples were dissolved in methanol, infused into the source at 60 ml/h and analyzed in the positive mode with the capillary heated to 210 °C and at a spray voltage of 4.0 kV.

2.7. Statistical analyses

All analyses were performed using the statistical program StatView® 5.0.1 (SAS Institute, 1998). Tests are two-tailed, the assumed level of significance is $P<0.05$ and means ± S.E. are reported in all cases. We used analyses-of-variance (ANOVA) to evaluate differences in the proportional composition of carotenoid pigments in diet, serum, liver,

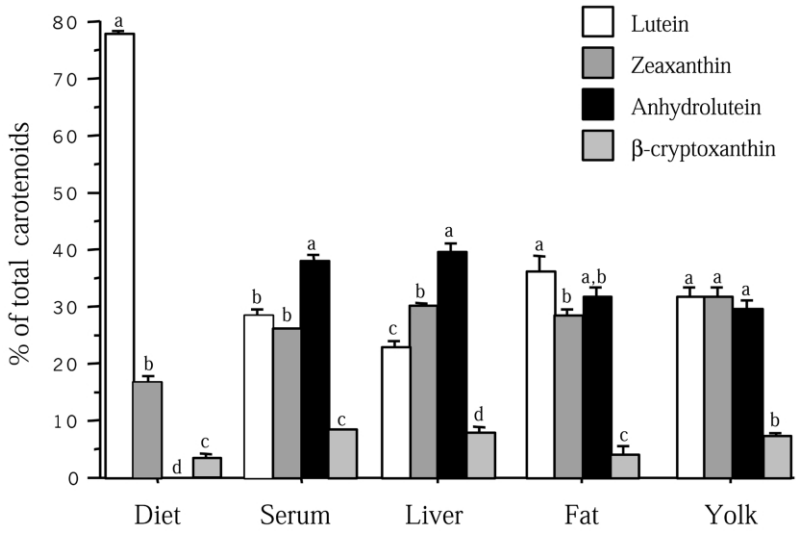

Fig. 1. Relative abundance of carotenoid pigments in zebra finch diet, plasma and tissue. The carotenoid profile of each sample was determined by conventional reverse-phase HPLC. Letters denote significant differences in carotenoid composition (within tissues only) as determined by post hoc paired comparisons (see Section 2).

adipose tissue and egg yolk. When general linear models yielded significant differences, we conducted post hoc analyses (Fisher's PLSD test) to determine particular differences in pigment composition within the five sample types. We also examined the proportional relationships among the pigments in finch blood using Fisher's *r*-to-*z* tests for Pearson's correlations.

3. Results

3.1. Dietary carotenoids

The commercial seed mix fed to zebra finches contained four primary carotenoid pigments: lutein, zeaxanthin, β-cryptoxanthin and β-carotene. The dihydroxy-carotenoid lutein was the major pigment in finch food, comprising over 75% of all dietary carotenoids, while β-cryptoxanthin and β-carotene together constituted only 5% of pigments in seed (Fig. 1). Analysis of saponified and unsaponified extracts yielded identical food-carotenoid profiles.

3.2. Plasma carotenoids

Zebra finches circulated three of the four dietary carotenoids in blood (lutein, zeaxanthin and β-cryptoxanthin; Fig. 2), but there was no detectable plasma β-carotene, perhaps due to efficient hepatic and duodenal conversion to vitamin A (Wyss et al., 2001). We also isolated a fourth, unidentified plasma carotenoid. We investigated the identity of this unknown pigment by comparing its HPLC retention time, UV-Vis absorption spectra and molecular mass (determined by mass spectrometry) to known metabolic derivatives of dietary carotenoids in human plasma (Khachik et al., 1992). This finch plasma pigment yielded λ_{max} = 448 and 476 nm (in the aforementioned HPLC mobile phase) and a molecular anion peak = 550. These data are consistent with the presence of an isomeric form of anhydrolutein, two of which are found in human plasma (anhydrolutein I and II; Khachik et al., 1995). HPLC analyses of this unknown carotenoid using a weaker solvent system (methanol/acetonitrile, 50:50, v/v) showed that it most closely matched the retention time of anhydrolutein II (2′,3′-anhydrolutein; standards provided by F. Khachik). We believe this is the first documented presence of 2′,3′-anhydrolutein in any avian species (hereafter referred to as anhydrolutein). We have also recently identified anhydrolutein in the blood of two related estrildid finch species that were captured from the wild (*Estrilda astrild* and *Sporaeginthus subflavus*; K. McGraw, J. Schuetz and R. Parker, unpublished data).

Anhydrolutein was the most abundant pigment in zebra finch plasma, comprising 38% of all blood carotenoids (Fig. 1). Lutein and zeaxanthin

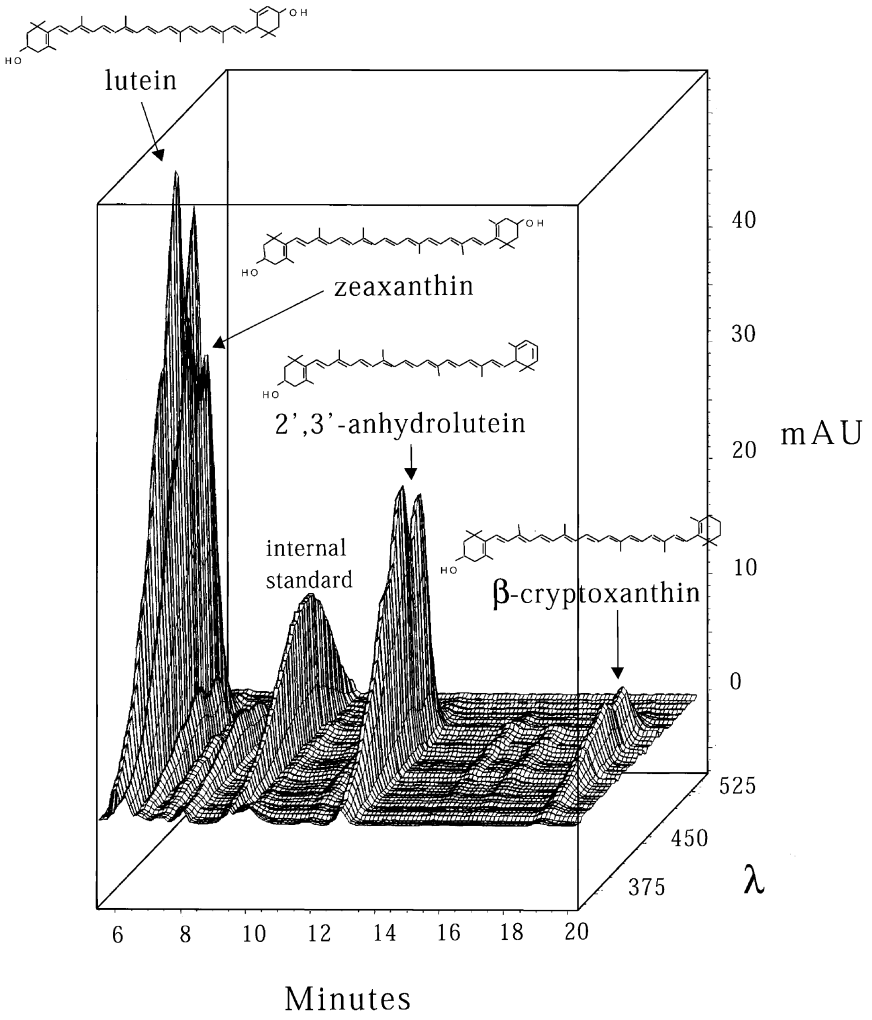

Fig. 2. Representative three-dimensional HPLC chromatogram for the carotenoid pigments present in zebra finch plasma. Lutein, zeaxanthin and β-cryptoxanthin were identified by comparison to authentic standards supplied by Roche Vitamins Co. (Parsippany, NJ), while anhydrolutein was identified by comparison to standards supplied by F. Khachik as well as by mass spectrometry (see Section 2).

were present in similar proportions (28 and 26%, respectively), while β-cryptoxanthin again occurred in low concentration (8% of total). Finch plasma was relatively enriched with zeaxanthin and β-cryptoxanthin compared to the diet, but contained proportionally less lutein than the food (Fig. 1). This suggests that lutein may be the primary substrate from which anhydrolutein is formed, and generates the prediction that individual birds who circulate a higher percentage of anhydrolutein in plasma will have comparatively less lutein remaining in circulation. In fact, birds whose plasma contained proportionally more anhydrolutein circulated significantly less lutein (Table 1). We also found a significant negative correlation between the relative amounts of plasma lutein and β-cryptoxanthin (Table 1, all other $P > 0.05$).

3.3. Tissue carotenoids

The carotenoids present in zebra finch liver, adipose tissue and egg-yolk matched those four pigments detected in plasma—anhydrolutein, lutein, zeaxanthin and β-cryptoxanthin. However, the proportional abundance of each carotenoid type differed between plasma and tissues. Like plasma, anhydrolutein was the major liver carotenoid (39% of total), but was not the primary pigment found

Table 1
Proportional relationships between blood carotenoid types in captive zebra finches

Plasma carotenoid pair	*r*	*Z*	*P*
% lutein,% zeaxanthin	−0.14	−0.68	0.50
% lutein,% anhydrolutein	−0.79	−5.17	<0.0001
% lutein,% β-cryptoxanthin	−0.67	−3.91	<0.0001
% zeaxanthin,% anhydrolutein	−0.37	−1.84	0.07
% zeaxanthin,% β-cryptoxanthin	0.14	0.69	0.49
% anhydrolutein,% β-cryptoxanthin	0.24	1.18	0.24

$n = 24$ birds in each comparison.

in body fat (32%) or yolk (29%; Fig. 1). Instead, lutein predominated in adipose tissue (35%), and lutein, zeaxanthin and anhydrolutein were present in equal proportions in yolk (31, 31 and 29%, respectively; Fig. 1). β-cryptoxanthin remained low and varied little across all tissues (4–8%; Fig. 1).

4. Discussion

Zebra finches are grass seed specialists (Zann, 1996), with millet comprising the majority of the seeds fed to and preferred by captive birds (e.g. Williams, 1996; Bradbury and Blakey, 1998). We isolated four major carotenoids from our millet-based finch food mix: lutein; zeaxanthin; β-cryptoxanthin; and β-carotene. This suite of pigments typically exists in non-esterified form in other bird seeds (McGraw et al., 2001) and has been isolated from the gut contents of a few colorful finch species in the wild (e.g. *Carpodacus mexicanus*, Inouye, 1999; *Carduelis tristis*; K. McGraw, unpublished data). Lutein and zeaxanthin are also major carotenoid components of plant-based diets in humans (Mangels et al., 1993). β-Cryptoxanthin and β-carotene are common in certain fruits and vegetables (Mangels et al., 1993) and in aquatic animals (e.g. fish, crustaceans) that may make up the diet of certain waterbirds (Simpson et al., 1981), but are less prevalent in seeds (Goodwin, 1980).

Birds, like other animals, absorb dietary carotenoids through the intestinal mucosa, where they are transferred to lipoprotein particles and transported through the blood to peripheral tissues in the body (Trams, 1969; Erdman et al., 1993; Parker, 1996). Among the primary diet pigments, zebra finches circulated lutein, zeaxanthin and β-cryptoxanthin in plasma. Lutein and zeaxanthin have been detected as major serum carotenoids, with β-cryptoxanthin a minor component, in a wide range of avian species (Slifka et al., 1999). β-Carotene was undetectable in blood samples, which is characteristic of birds and fish that poorly utilize non-polar carotenoids, like the carotenes, and instead preferentially accumulate polar hydroxy- and ketocarotenoids (Scheidt, 1998; Slifka et al., 1999; but see Royle et al., 1999).

We also isolated a fourth plasma carotenoid, 2′,3′-anhydrolutein, that was the most concentrated of all serum pigments and had never before been described in birds. Anhydroluteins are rare among both plants and animals (Barua and Das, 1975), serving only as minor carotenoid constituents in: squash (Khachik and Beecher, 1988; Khachik et al., 1988) and black paprika (Deli et al., 1994); the plasma (Khachik et al., 1992) and skin (Wingerath et al., 1998) of humans; and the liver oils of a freshwater fish (Barua and Das, 1975). This pigment was absent from both the saponified and unsaponified diet samples in our study, indicating that anhydrolutein is physiologically derived from dietary carotenoids in zebra finches. In humans, anhydrolutein is presumed to be a dehydration product of dietary lutein (Khachik et al., 1992, 1995). Because the relative abundance of lutein varied inversely with anhydrolutein in finch plasma, and because we found proportionally less lutein in finch plasma than in the diet, it seems that lutein is the likely substrate for anhydrolutein biosynthesis in zebra finches as well.

We also determined the carotenoid content of various finch tissues, which allowed us to investigate potential production sites of anhydrolutein. In vitro exposure of lutein to acidic conditions forms anhydrolutein by-products (Budowski et al., 1963; Khachik et al., 1995), so it has been suggested that acidic gastric juice in the stomach catalyzes this dehydration reaction in vivo (Khachik et al., 1995). First, we first collected sections of the digestive system (crop, esophagus, proventriculus,

gizzard, small intestine and large intestine) and determined the carotenoid content along the GI tract for a single animal. We found no evidence of anhydrolutein in the crop or proventriculus, only trace amounts in the gizzard, but substantial levels in the duodenum (carotenoids were absent in bile and feces). This pattern provides no support for the chemical formation of anhydrolutein in the stomach. Instead, we might expect tissues downstream from the stomach, such as the duodenum, to manufacture anhydrolutein, where enzymes that metabolize β-carotene are present in developing chicks (*Gallus domesticus*; Tajima et al., 2001). The liver has also been proposed as a major site of carotenoid bioconversion in birds (Brush, 1990; Tajima et al., 2001). The fact that the liver was enriched with anhydrolutein and was comparatively deficient in lutein, relative to plasma and other tissues, suggests that this organ may also be a site of anhydrolutein synthesis in zebra finches.

We are currently investigating the physiological fate and function of anhydrolutein in this species. When dietarily supplemented with anhydrolutein III (3′,4′-anhydrolutein), rats (Savithry et al., 1972), mice (Budowski and Gross, 1965), chicks (Budowski et al., 1964) and fish (Barua and Das, 1975) all convert this carotenoid into vitamin A_2—the basis for visual pigments in certain vertebrates (Provencio et al., 1992; Loew and Sillman, 1993). However, it is unclear if the form of anhydrolutein in zebra finches is a viable precursor of this vitamin. This carotenoid may also be an intermediate in the pathway that forms more reduced lutein metabolites (e.g. β-cryptoxanthin), which might occur through the removal of the hydroxyl group in position 3 of the ε-ionone end-ring of lutein. This potential precursor-product relationship between lutein and β-cryptoxanthin could explain the strong negative correlation between these two carotenoids in plasma. Last, the role of anhydrolutein as a pigmentary colorant of the finch integument (beak, legs) should be investigated further. Although we have yet to characterize the carotenoids present in finch bills because of the high degree of pigment esterification in this tissue (resulting in poor chromatographic resolution), it appears that red, 4-oxo-carotenoids are contained within their reddish-orange bills (R. Stradi, pers. comm.). Thus, anhydrolutein may serve as a valuable precursor for the formation of red ketocarotenoids and may help explain both within- and between-sex differences in carotenoid-derived ornamental coloration in these birds.

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