Carotenoid Accumulation in the Tissues of Zebra Finches: Predictors of Integumentary Pigmentation and Implications for Carotenoid Allocation Strategies

Kevin J. McGraw*
Matthew B. Toomey†
School of Life Sciences, Arizona State University, Tempe, Arizona 85287

Accepted 8/30/2009; Electronically Published 11/24/2009

Online enhancement: appendix table.

ABSTRACT

Carotenoid pigments produce the bright yellow to red ornamental colors of many animals, especially birds, and must ultimately be derived from the diet. However, they are also valuable for many physiological functions (e.g., antioxidants, immunostimulants, photoprotection, visual tuning, yolk nourishment to embryos), and as a result they are present in numerous internal body tissues (e.g., liver, adipose tissue, retina) whose carotenoid types and amounts are rarely studied in the context of color acquisition. Because male and female animals typically place different priorities on fitness-enhancing activities (e.g., gametic investment in females, sexual attraction in males), carotenoid allocation may track such investment patterns in the two sexes, and we can test for such sex-specific priorities of carotenoids by assessing body-tissue distributions of these pigments. We used high-performance liquid chromatography to identify and quantify carotenoid pigments from the plasma, liver, adipose tissue, and retina as well as the beak and legs of male and female zebra finches (*Taeniopygia guttata*), a species in which males display sexually attractive, red, carotenoid-based beak coloration and females also display some (albeit a less rich orange) beak color. To our knowledge, this is the first study of the predictors of carotenoid-based leg coloration—another potentially important visual signal—in this species. The same suite of dietary (e.g., lutein, zeaxanthin, β-cryptoxanthin) and metabolically derived (e.g., dehydrolutein, anhydrolutein) yellow and orange carotenoids was present in plasma, liver, and adipose tissue of both sexes. Retina contained two different metabolites (astaxanthin and galloxanthin) that serve specific functions in association with unique photoreceptor types in the eye. Beaks were enriched with four red ketocarotenoid derivatives in both sexes (α-doradexanthin, adonirubin, astaxanthin, and canthaxanthin), while the carotenoid profile of legs was similar to that of plasma/liver/adipose tissue but with the additional presence of canthaxanthin. Sex differences in beak coloration were attributable to different concentrations of all four red ketocarotenoids. Males also had more colorful legs than did females, and this color difference was due to the increased presence of canthaxanthin in males. Males had higher carotenoid concentrations in plasma and retina than did females, but females had higher carotenoid concentrations in liver and adipose tissue than did males. These patterns are consistent with the apparently different life-history strategies employed for carotenoids by adult males and females, with females prioritizing future access to carotenoids (in tissue stores for egg production) and males prioritizing current access (in circulation, for maintaining bright color and/or health).

Introduction

Interest in carotenoid pigments has accelerated in recent years among evolutionary biologists (McGraw 2006). Studying the abundance, use, and consequences of these diet-derived, health-enhancing, and color-producing molecules has provided valuable insights into many aspects of life-history evolution in animals (Blount 2004). For example, trade-offs in somatic, gametic, and sexual investments are often manifested in the form of unique carotenoid pigment allocations to health versus eggs versus body coloration in birds (Faivre et al. 2003; Peters et al. 2004; Hargitai et al. 2005). Because the sexes can differ dramatically in the relative value of such life-history investments (e.g., gametic investment in females, sexual attraction in males), we might expect very different "priorities" to be given to carotenoids by males and females within individuals at any given time or over the different life-history stages.

One means by which to study comparative functional apportioning of carotenoids in animals is to examine the distribution of carotenoids throughout various tissues in the body. Carotenoids can be devoted to particular tissues for specific functions. Beyond the obvious pigmentary (and perhaps photoprotective) role of carotenoids in the integument (e.g., feathers, beak, legs), carotenoids in internal tissues such as adipose or liver may be used as "storage depots" (Negro et al. 2001) for access at later critical time periods (e.g., migration, health stressors, molt, reproduction). Carotenoids deposited in egg

* Corresponding author; e-mail: kevin.mcgraw@asu.edu.
† E-mail: matthew.toomey@asu.edu.

Physiological and Biochemical Zoology 83(1):97–109. 2010. 1522-2152/2010/8301-8267$15.00
DOI: 10.1086/648396

yolk by laying females may be derived largely from mobilized fat stores, especially in capital breeders who draw on adipose reserves for yolk nutrients (Moreno 1989; Speake et al. 1999). Carotenoids are also deposited in the avian retina (Wald and Zussman 1937), where they occur in oil droplets that are coupled to specific photoreceptors (Goldsmith et al. 1984) and protect cone cells from photodamage (Thomson et al. 2002*a*, 2002*b*) as well as tune their spectral sensitivities (Vorobyev 2003). To date, most attention has been paid to the qualitative characteristics of avian retinal carotenoids (inferred from microspectrophotometry; Cuthill 2006) and very little to the understanding of the chemical composition of, variation in, and biological predictors of carotenoids in the avian retina.

If the sexes do place different priorities on the carotenoids they acquire, then on the basis of their life-history strategies we should expect to see very different patterns of carotenoid accumulation throughout the body. Therefore, we examined the types and amounts of carotenoid pigments present in six main fluids and tissues—plasma, liver, adipose, retina, beak, and legs—in the zebra finch (*Taeniopygia guttata*). This is an ideal species for studying carotenoid allocation among body tissues because (1) males display sexually selected red carotenoid-based beak coloration (whereas females display reduced orange beak coloration that is not directionally selected; Burley and Coopersmith 1987); (2) females invest heavily in reproduction in their short lifetimes, producing two to three clutches a year of about five eggs each for 3–5 yr in the wild (Zann 1996); and (3) the sexes do not differ significantly in food (carotenoid) intake (McGraw et al. 2003), hence making any body-tissue differences in carotenoid content a product of intrinsic (e.g., physiological, morphological) processes.

We asked the following specific questions in our research. (1) What different carotenoids are found in these various body tissues in zebra finches? McGraw et al. (2002) documented carotenoid profiles for adipose, yolk, liver, and plasma in male and female zebra finches but did not investigate sex differences for these tissues or describe carotenoids from retina, beak, or leg in either sex. (2) Do the sexes differ in carotenoid types and concentrations in each tissue type? (3) Do carotenoid levels in different body tissues correlate with concentrations found in the integument (as well as beak and leg coloration)? Several previous studies of zebra finches have shown that plasma carotenoid levels significantly and positively predict beak coloration (McGraw and Ardia 2003; McGraw et al. 2003; McGraw and Parker 2006), but these studies did not examine any other tissue sources and did not directly measure carotenoid pigments in the beak. In molting house finches (*Carpodacus mexicanus*), for example, liver carotenoid stores are equally predictive of plumage coloration as are plasma concentrations (McGraw et al. 2006*b*). We also are the first to investigate the nature of leg coloration in this species, which is bright orange but has not been considered previously in any mechanistic or functional study of coloration in zebra finches. (4) Do concentrations of one type of carotenoid correlate positively with all other types accumulated in a particular body region? Because carotenoids come in different forms (e.g., carotenes vs. xanthophylls), they may not accumulate in the same fashion or serve identical functions, as is true for the specialized accumulation of β-cryptoxanthin for red color development in house finches (McGraw et al. 2006*c*). Previous work on zebra finches suggested that all carotenoid types were equally accumulated and important for color development, although again this analysis was only performed for plasma carotenoids (McGraw et al. 2003). (5) Do birds that have high concentrations of carotenoids in one tissue type also have high levels in all other internal tissues? Some animals may simply have a high affinity for carotenoid uptake and accumulate high carotenoid levels throughout the body's fluids and tissues; alternatively, carotenoids may be differentially allocated to or drawn from particular tissues (e.g., from plasma to storage sites), which would lead to negative correlations between different pools (Negro et al. 2001).

Methods

In June–July 2006, we studied carotenoids in 14 adult female and 15 adult male zebra finches that were housed individually in small cages (39 cm tall × 28 cm long × 21 cm wide) in animal-approved indoor rooms (males in a greenhouse, females in a windowless room) on the campus of Arizona State University (see McGraw 2005 for more husbandry details). All individuals had previously completed one breeding attempt 18 mo before and were at least 3 yr old. We attempted to house all birds in nonbreeding conditions by not providing them with nest material or cups, but there were a few females who laid eggs and a few males who exhibited singing behavior, suggesting that there was some variation in both sexes in physiological and behavioral breeding status. We fed the finches a seed-mix diet that contained predominantly xanthophylls (means ± SDs reported based on triplicate analyses; lutein concentration = 4.17 ± 0.60 μg g^{-1}, zeaxanthin concentration = 0.96 ± 0.27 μg g^{-1}) but also had small amounts of β-cryptoxanthin (0.04 ± 0.01 μg g^{-1}) and β-carotene (0.14 ± 0.04 μg g^{-1}). Light cycles mimicked outdoor lighting conditions in Arizona. All birds appeared healthy for months before this study, showing no signs of parasitic infection (e.g., weight loss, lethargy).

For each bird, a small blood sample (<100 μL) was drawn from the wing vein for plasma collection (sensu McGraw et al. 2002) and beak/leg coloration was scored using digital photography (sensu McGraw 2007). We used hue (i.e., true color, measured in integer units around a 360° color wheel, with red set at 0°) to measure carotenoid-based beak and leg coloration, both of which were highly repeatably scored from the two photographs taken per body region per bird (beak: $R_i = 0.99$, $F_{1,28} = 22.6$, $P < 0.0001$; leg: $R_i = 0.86$, $F_{1,28} = 14.0$, $P < 0.0001$). Saturation is another of the tristimulus scores that can capture variation in carotenoid concentration of some colorful tissues (Andersson and Prager 2006), but it was significantly correlated with hue in both the legs and beaks (all $r < -0.59$, all $P < 0.007$), and thus we focused on only one metric here (sensu McGraw et al. 2004, 2005, 2006*a*; McGraw and Parker 2006). Birds were then quickly killed, and adipose (from the clavicular region; also referred to as "fat tissue" throughout),

liver (right lobe), and retinal (left eye) tissue were immediately dissected (see Toyoda et al. 2002 for details of retinal dissection). Carcasses were frozen at −20°C for several weeks, after which time we shaved hardened outer tissue/scales from the beak and legs.

Following McGraw et al. (2002), we extracted carotenoids from plasma using organic solvents and from all tissues using a mortar and pestle in the presence of solvent (5–6 mL hexane : *tert* butyl methyl ether, 1 : 1, v/v). We extracted carotenoids from 10 μL of plasma and ~0.01–0.015 g of fat, 0.03–0.4 g of liver, 0.02–0.04 g of retina, 0.002–0.01 g of beak shavings, and 0.001–0.007 g of leg scales. Ground tissue/deproteinated plasma and solvent were centrifuged, and the supernatant was recovered for carotenoid analysis. For plasma and liver extracts only, we evaporated the solvent to dryness in a fresh tube and resuspended the residue in mobile phase (methanol : acetonitrile : dichloromethane, 42 : 42 : 16, v/v/v) for high-performance liquid chromatography (HPLC) analysis (see below). All other samples (adipose, retina, beak, legs) required saponification to rid them of fatty-acid esters and other lipids that disrupt HPLC elution. Carotenoids of differing degrees of oxygenation are differentially sensitive to base-catalyzed hydrolysis (Goodwin 1980), so we developed two saponification methods (one targeting xanthophyll recovery and another for ketocarotenoid recovery) to optimally saponify all carotenoid types in any given sample (Toomey and McGraw 2007). We used the ketocarotenoid method on beak; the xanthophyll method on leg, adipose, and liver; and both methods on the retina, based on the dominant carotenoid classes that these tissues contain (as determined in previous work, e.g., McGraw et al. 2002; K. J. McGraw and M. B. Toomey, unpublished data). Both methods were performed on the retinal samples by splitting extracts in two before evaporation to dryness. The only difference between our two saponification procedures is the strength of the base added. For the xanthophyll method, we added 1 mL of 0.2 M methanolic potassium hydroxide (KOH) to the pigment residue; for the ketocarotenoid method, we added 1 mL of 0.02 M methanolic KOH. Retinal samples were saponified for 6 h and all other samples were held in the dark at room temperature overnight. We then added 1 mL of saturated sodium chloride and 2 mL of distilled H_2O and vortexed the solution for 5 s after the addition of each liquid. We finished the extraction by adding 3 mL of hexane : *tert* butyl methyl ether (1 : 1) to each tube and inverting it back and forth for 1 min to mix the layers thoroughly. At this point, all tubes were centrifuged, and their supernatants were retrieved and dried down as described above for HPLC analysis.

HPLC analyses of plasma, adipose, and liver carotenoids follow those in McGraw et al. (2006*c*), using a Waters 2695 instrument (Waters, Milford, MA). Because of the presence of ketocarotenoids in beak, leg, and retina samples, we slightly modified our analytical method for these tissues. First, the HPLC column (Waters YMC Carotenoid column, 5 μm, 4.6 mm × 250 mm) was pretreated with 1% orthophosphoric acid in methanol for 30 min at 1 mL min^{-1} (sensu Vecchi et al. 1987). Second, we altered solvent composition and flow rate to optimize separation of different ketocarotenoids. At a constant flow rate of 1.2 mL min^{-1}, we first used isocratic elution with 48 : 48 : 4 (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 through 25 min, and finishing with a return to the original isocratic conditions from 25–35 min. Carotenoid types (Fig. 1) were identified by comparison to authentic standards acquired from CaroteNature (Lupsingen, Switzerland), Sigma-Aldrich (St. Louis, MO), DSM (Heerlen, Netherlands), Dr. Fred Khachik (University of Maryland), and Dr. Riccardo Stradi (University of Milan) or to data from the literature (Toomey and McGraw 2007). We used external standard curves to quantify concentrations of each carotenoid type. We did not sample and weigh whole livers, beaks, etc., for this study, so we could not compute total carotenoid amounts per tissue type (but see estimations provided in Table A1 in the online edition of *Physiological and Biochemical Zoology*).

Results

Carotenoid Profiles in Different Tissues

Carotenoid profiles were qualitatively similar in the two sexes. Plasma, liver, and adipose samples generally contained the same types of carotenoids: lutein, zeaxanthin, 3′-dehydrolutein, 3′-anhydrolutein, and β-cryptoxanthin (Fig. 1). Anhydrolutein and dehydrolutein are metabolically derived, likely in the gastrointestinal tract, from dietary lutein (McGraw et al. 2002; McGraw and Schuetz 2004). Several (cis)lutein isomers were also present in adipose, likely formed during saponification. β-carotene was present only in liver and in plasma, where it was very dilute.

Beak, legs, and retina each had different carotenoid profiles. Four metabolically derived ketocarotenoids—α-doradexanthin, adonirubin, astaxanthin, and canthaxanthin—were present in beak tissue along with two xanthophylls (lutein and anhydrolutein; Fig. 1). Leg carotenoids were very similar to those in plasma/liver/adipose but also contained canthaxanthin and lacked β-cryptoxanthin. Retina contained one apocarotenoid (galloxanthin, a metabolically derived form found nowhere else in the body), one ketocarotenoid (astaxanthin), two xanthophylls (epilutein and zeaxanthin), and one carotene (which we could not identify and as such labeled ?-carotene; Fig. 1); this profile is similar to those in retinas of other avian species (e.g., Japanese quail *Coturnix japonica*, Toomey and McGraw 2007; house finch, Toomey and McGraw 2009).

Carotenoids varied in concentration both among and within tissues. Among tissues (considering total carotenoid concentration and excluding plasma because it is measured in volume, not mass), beak was the most carotenoid-enriched tissue in zebra finches, followed by leg, adipose, retina, and liver (Fig. 2*A*). Within individual fluid/tissue types, lutein was the most concentrated of the carotenoids in plasma, liver (along with anhydrolutein), and fat (Fig. 2*B*). β-cryptoxanthin and β-carotene were always the least concentrated pigments in the tissues in which they were found (Fig. 2*B*). Surprisingly, orange

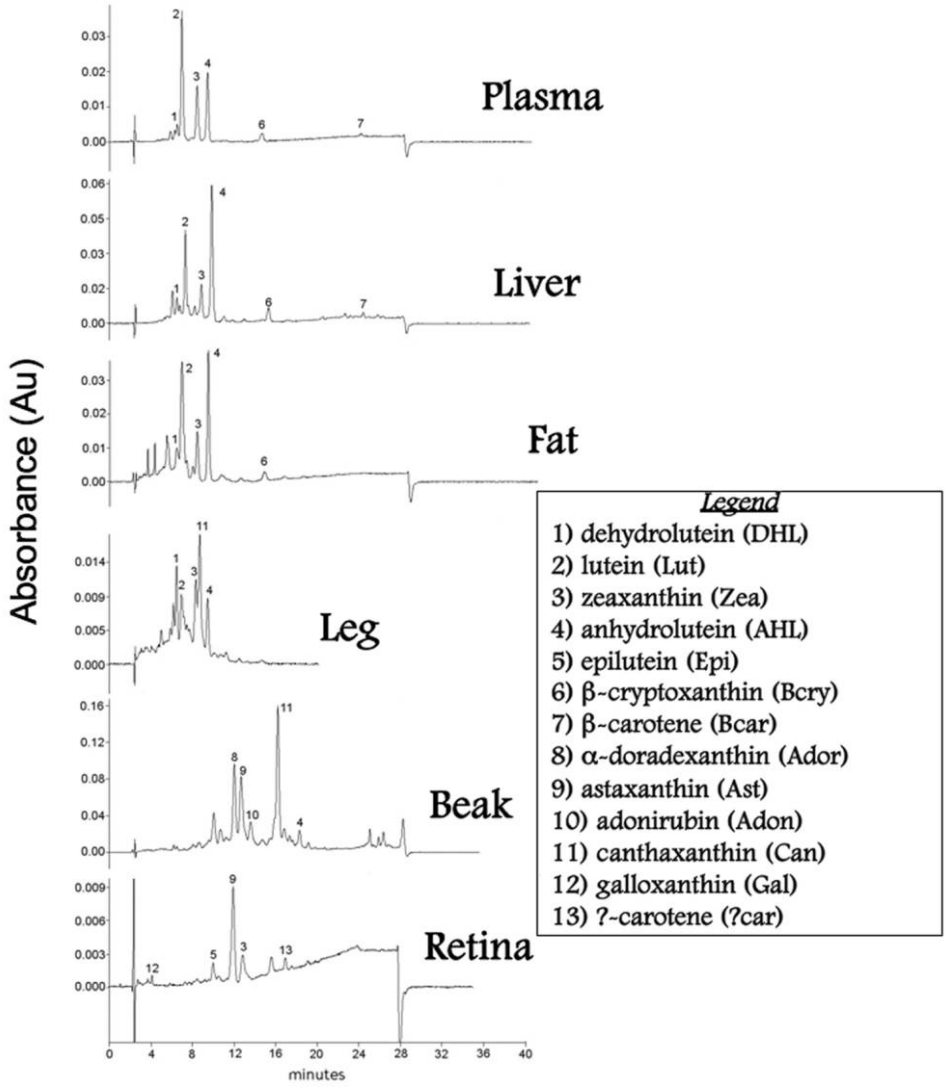

Figure 1. Representative two-dimensional high-performance liquid chromatography (HPLC) chromatograms showing the carotenoid complement from each fluid/tissue analyzed in zebra finches. Time (along the *X*-axis) runs from 0–40 min in all chromatograms; the *Y*-axis denotes arbitrary absorbance units measured by the HPLC. All traces were taken at $\lambda = 448$ nm. Note for retina, however, that galloxanthin (12) absorbs light maximally at 375 nm and thus occurs at higher relative concentrations than is shown here. Retention times for certain carotenoids differ across chromatograms because different HPLC conditions were used to analyze beak, leg, and retina than plasma, liver, and adipose tissue (see "Methods"). Abbreviations for all carotenoid names are shown here and will be used in subsequent figures. Lutein isomers in fat (see text for more information) eluted earlier than all other carotenoids and were summed for statistical analyses (see Figs. 2, 3).

legs were enriched mostly in yellow xanthophylls (>80% of total), with the one orange ketocarotenoid (canthaxanthin) being the least concentrated of the types present (Fig. 2*B*). The four ketocarotenoids were dominant in beak (~85% of total; Fig. 2*B*). Retina contained mostly galloxanthin (most concentrated at ~60% of total) and astaxanthin (~20% of total; Fig. 2*B*).

For estimates of total carotenoid quantities in internal tissues, which may give a sense for their relative potential to contribute to carotenoid supplies in beak and legs, see Table A1.

Sex Differences in Tissue Carotenoid Accumulation

We used MANOVAs to test for sex differences in individual carotenoid concentrations within tissue types. As we have shown previously (McGraw et al. 2003), male zebra finches circulated a significantly higher concentration of all carotenoids through plasma than did females (multivariate comparison: Wilks' $\lambda = 0.27$, $P < 0.0001$; $P < 0.007$ for all univariate comparisons of individual carotenoids; Fig. 3). Male beaks also contained a higher concentration of all carotenoids (Wilks' $\lambda = 0.13$, $P < 0.0001$; $P < 0.0001$ for all individual carotenoids;

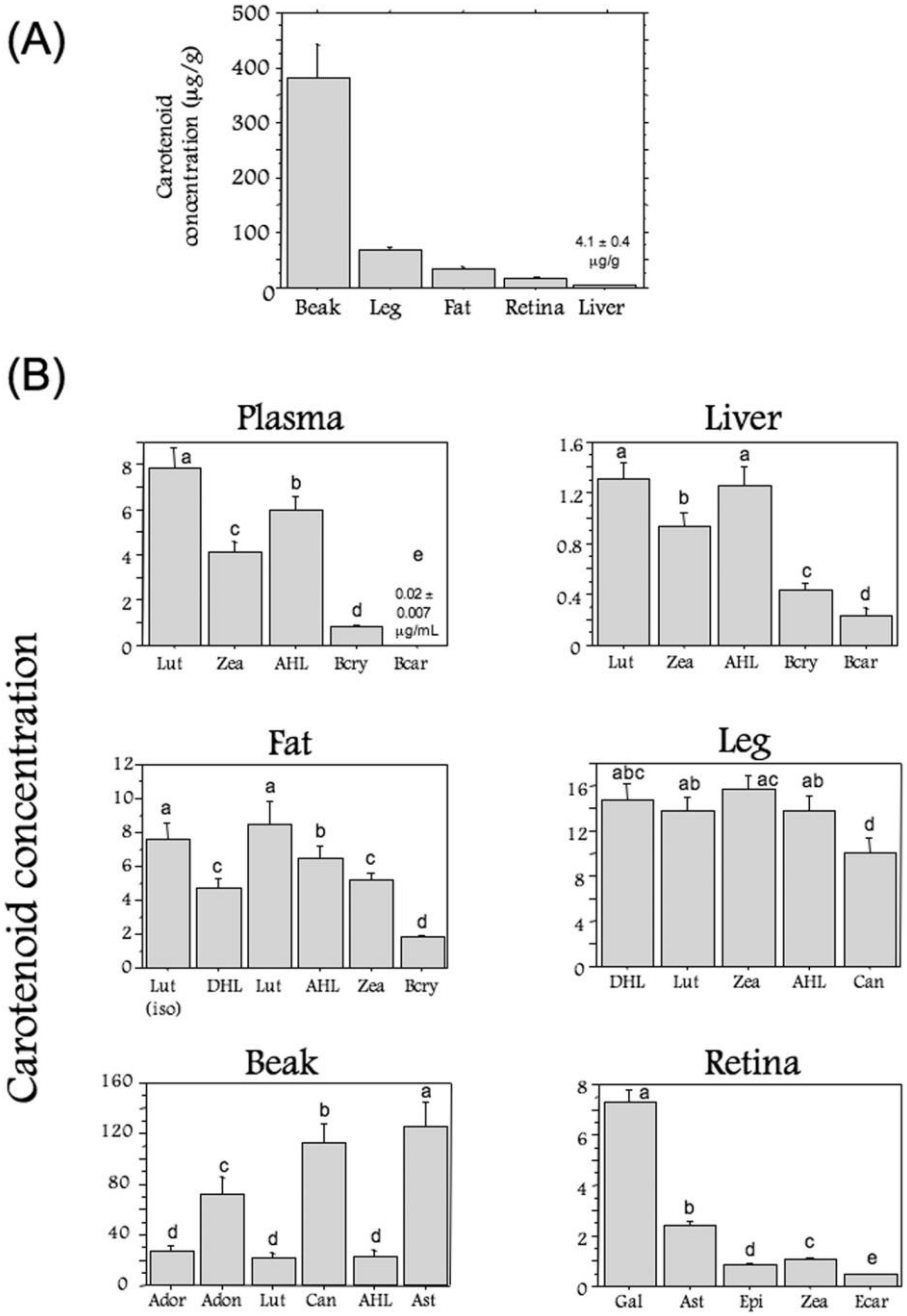

Figure 2. *A*, Differences in total carotenoid concentrations among tissue types in zebra finches. For comparison (although in different units), mean ± SEM plasma carotenoid levels for birds in this study was 18.8 ± 1.9 μg mL^{-1}. *B*, Proportional composition of carotenoids within tissue types. Units for plasma carotenoid concentrations are in μg mL^{-1}, but for all other tissues they are μg g^{-1}. We ran repeated-measures ANOVA to examine differences in total carotenoid concentration among tissue types in *A* and found that all groups differed from each other (full model: Greenhouse-Geisser corrected $F_{1.019, 25.471} = 32.36$, $P < 0.0001$; as well, $P < 0.0001$ for all post hoc pairwise comparisons among tissues). We did the same for all carotenoid types within a tissue in *B*, with all full models resulting in $P < 0.001$; here, unshared letters denote statistically significant post hoc differences in concentration (within a panel). All data shown here are pooled for the sexes because our aim with these analyses was to be descriptive (not sex specific) and because males and females largely showed the same patterns in these analyses anyway (also see Fig. 3, where similarities in the relative abundance of carotenoid types within a tissue and in total carotenoid concentrations among the tissue types is visually evident for males and females). Dehydrolutein was a very minor component in plasma and liver and thus was lumped together with "lutein" in statistical analyses for these tissues.

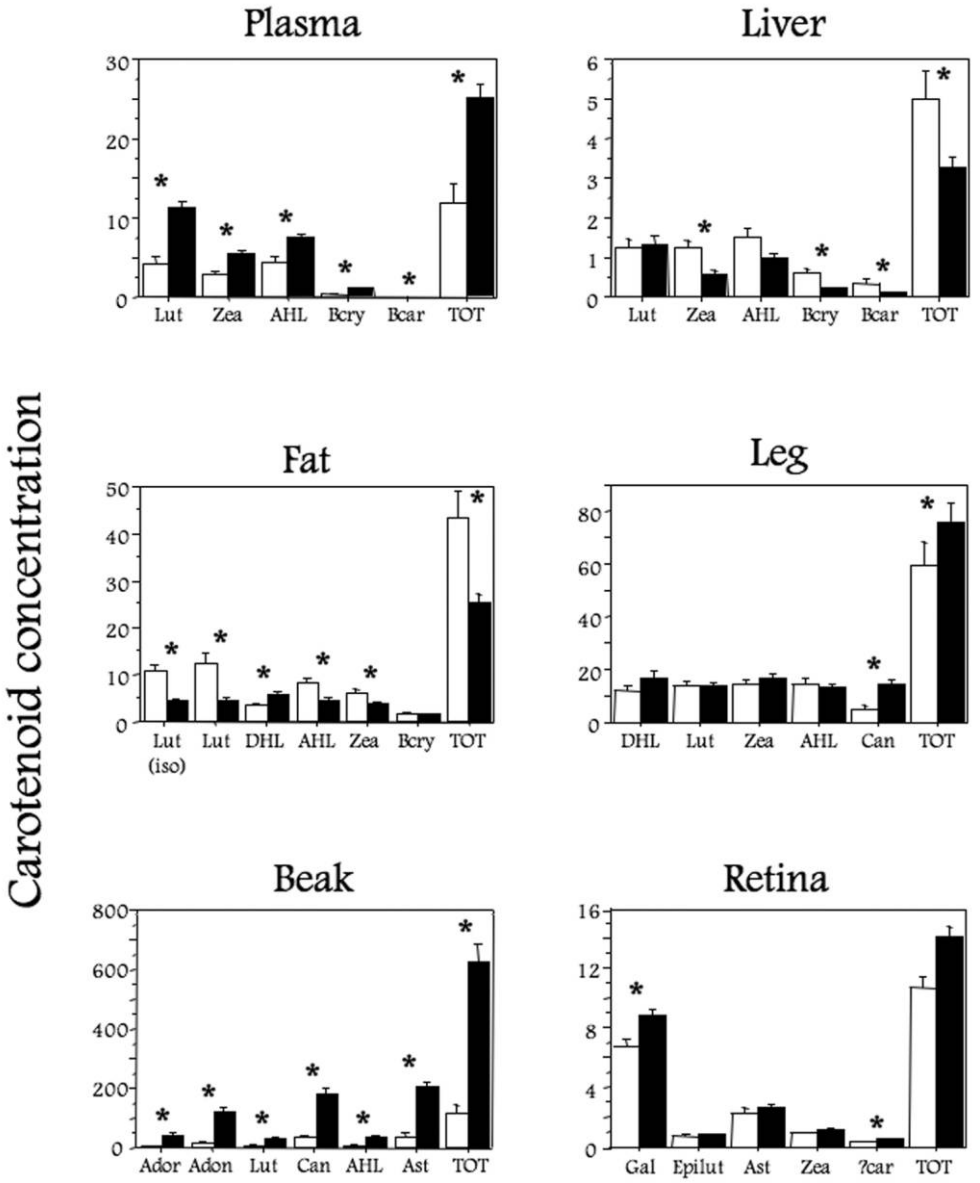

Figure 3. Sex differences in carotenoid concentrations among different body fluids/tissues in zebra finches. Males = filled bars; females = unfilled bars. Asterisks denote significant sex differences (see text for statistics). See Figure 2 for additional details.

Fig. 3) than did those of females (as expected, because male beaks are more colorful). Legs of males and females differed in the concentration of only one carotenoid, canthaxanthin (Wilks' $\lambda = 0.38$, $P < 0.0001$; canthaxanthin: $F_{1,26} = 16.14$, $P < 0.0001$; all other $P > 0.06$; Fig. 3); this is probably the reason why we found that males had more orange legs than did females (male hue = 15.5° ± 0.8°; female hue = 21.4° ± 0.6°; $F = 34.5$, $P < 0.0001$). Males also had higher levels of galloxanthin ($F_{1,15} = 8.36$, $P = 0.011$) and ?-carotene ($F_{1,15} = 10.85$, $P = 0.005$) in retina than did females (Wilks' $\lambda = 0.52$, $P = 0.15$; $P > 0.07$ for all other carotenoids; Fig. 3).

In body fat (Wilks' $\lambda = 0.36$, $P = 0.001$) and liver (Wilks' $\lambda = 0.17$, $P < 0.0001$), however, females had significantly higher carotenoid concentrations than did males. This was true for zeaxanthin ($F_{1,25} = 14.56$, $P = 0.001$), β-cryptoxanthin ($F_{1,25} = 27.59$, $P < 0.0001$), and β-carotene ($F_{1,25} = 5.22$, $P = 0.031$) in liver (all other $P > 0.07$). It was also the case for lutein isomers ($F_{1,26} = 18.13$, $P < 0.0001$), lutein ($F_{1,26} = 6.86$, $P = 0.001$), zeaxanthin ($F_{1,26} = 9.93$, $P = 0.004$), and anhydrolutein ($F_{1,26} = 7.95$, $P = 0.009$) in adipose tissue ($P = 0.74$ for β-cryptoxanthin), although for one fat carotenoid, dehydrolutein, levels were higher in males than in females ($F_{1,26} = 13.68$, $P = 0.001$; Fig. 3).

Pigment and Tissue Predictors of Integumentary Carotenoids/Coloration

Separately in both sexes, we first compared beak and leg hue with carotenoid concentrations in each tissue using Pearson's

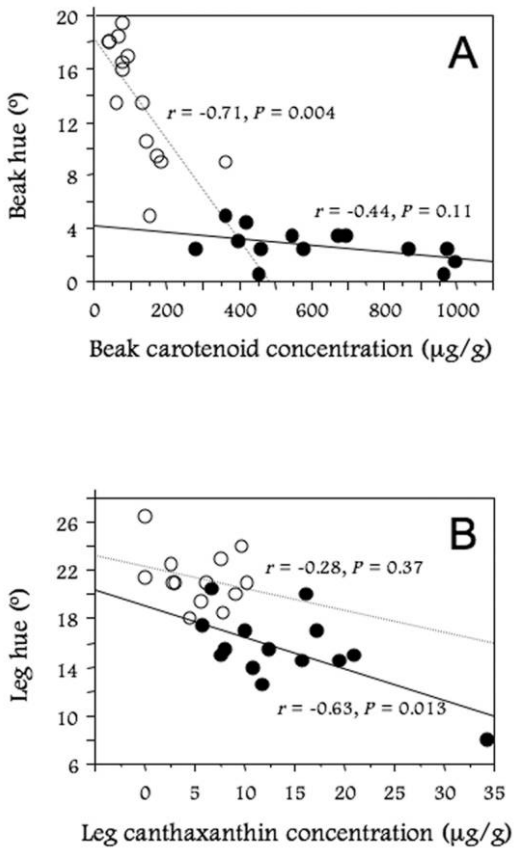

Figure 4. Sex-specific correlations between *A*, beak carotenoid concentration and hue and *B*, leg carotenoid concentration and hue. Filled circles and solid lines = males; open circles and dashed lines = females. Even if the point on the bottom right of *B* is removed, the relationship is still statistically significant ($r = -0.62$, $P = 0.01$).

product-moment correlations. Because concentrations of individual beak carotenoids were so highly intercorrelated (see more below) and differed so consistently between the sexes (as above), we used only total carotenoid concentration in beak analyses. In females, we found a highly significant negative correlation between beak hue and beak carotenoid concentration; that is, female beaks that were redder contained more carotenoids (Fig. 4*A*). In males, the same directional pattern was evident but was not statistically significant (Fig. 4*A*), and this may be due to the relative invariance in beak colors in our particular sample of males (spanning less than 5 hue units, compared with females that spanned nearly 15 units; hue variation of unmanipulated males in previous studies was >6 units; McGraw and Parker 2006; McGraw et al. 2006*a*).

Leg hue did not correlate significantly with total leg carotenoid concentration, either in males ($r = -0.42$, $P = 0.14$) or in females ($r = 0.19$, $P = 0.54$), although the pattern was in the predicted direction for males and was similar in magnitude to the beak color/carotenoid relationship for males. Canthaxanthin, however, was the specific carotenoid in legs that we presumed created the observed sex difference in leg color, so we compared leg canthaxanthin concentrations with leg hue and found in males, but not females (although again in the same direction), that redder legs contained significantly more canthaxanthin (Fig. 4*B*). No other correlations between leg hue and individual xanthophyll concentrations in legs were significant in males or females (all $P > 0.29$). We also analyzed integument hues in relation to the proportion of red ketocarotenoids in each respective tissue and uncovered the same statistical patterns for percent beak ketocarotenoids and percent leg canthaxanthin (data not shown) as described here for raw ketocarotenoid concentrations.

Our next aim was to understand what tissue sources of carotenoids were key for accumulating high concentrations of carotenoids in the integument. We were also curious whether accumulating high concentrations of carotenoids in one endogenous tissue meant that other tissues were deficient or were also highly enriched. Thus, we compared total carotenoid concentrations among all six tissue/fluid types studied separately for males and females (Table 1). Again, this was the most straightforward approach, as opposed to comparing concentrations of all individual carotenoid types against one another (which would have amounted to running over 600 correlations), because concentrations of different carotenoids were consistently positively correlated with one another within tissue types (see below). The first pattern of note in Table 1 is that nearly all correlation coefficients are positive (26 of 30; sign test, $P < 0.0001$). The only significant negative relationship between tissue carotenoid concentrations was between plasma and fat in females, such that females with low carotenoid concentrations in fat had high levels in plasma (Table 1). There were few significant patterns that showed up in both sexes. The only example of this was that males and females who deposited more carotenoids in beak had more carotenoids circulating through plasma and in liver (Table 1). In males, those individuals who had high carotenoid levels in fat also had high levels in leg and retina tissue (Table 1). In females, plasma and liver carotenoid levels were closely tied, as were retinal levels to beak and liver (Table 1). It was noteworthy that there was not a strong relationship between beak and leg carotenoids; the same was true for beak hue and leg hue in both sexes, although both correlations were positive (both $r < 0.4$, $P > 0.18$).

Table 1: Intercorrelations among total carotenoid concentrations in all tissues/fluids analyzed

	Plasma	Liver	Fat	Leg	Beak	Retina
Plasma		.76*	−.53*	−.13	.49*	.34
Liver	.29		−.38	.01	.68*	.58*
Fat	−.08	.21		.36	.15	.17
Leg	.04	.28	.51*		.30	.13
Beak	.54*	.64*	.28	.46		.70*
Retina	.25	.13	.52*	.34	.49	

Note. Data for females are shown in the upper right quadrant of the table and data for males are in the lower left quadrant.

* $P < 0.05$.

Table 2: Intercorrelations among concentrations of carotenoid types within plasma

	Lutein	Zeaxanthin	Anhydrolutein	β-Cryptoxanthin	β-Carotene
Lutein		.89*	.94*	.86*	.29
Zeaxanthin	.90*		.91*	.88*	.28
Anhydrolutein	.85*	.85*		.91*	.43
β-Cryptoxanthin	.24	.28	.27		.31
β-Carotene	.21	.22	.29	.46	

Note. Data for females are in the upper right quadrant and data for males are in the lower left quadrant. Because this was an exploratory study and we were interested in general patterns, we followed recommendations in the recent literature and did not employ a Bonferroni correction to adjust α levels for multiple tests (sensu Moran 2003; Garamszegi 2006; Montgomerie 2006).

* $P < 0.05$.

Intercorrelations of Carotenoid Concentrations within Different Body Tissues

We previously reported strong positive intercorrelations among all carotenoid types within plasma (McGraw et al. 2003), and here we show that this generally holds for all tissue types (Tables 2–5). Of the 136 correlations run, only two of them were negative (sign test, $P < 0.0001$), although neither was statistically significant. Of the positive correlations, 109 of 134 were, in turn, statistically significant (sign test, $P < 0.0001$). Patterns were strong in both sexes among the beak pigments (all $r > 0.77$, all $P < 0.0004$) but weaker for the leg pigments (all $r > 0.06$, all $P < 0.03$). The few instances where correlations were not consistently significant in plasma, liver, fat, and retina occurred for the less polar carotenoids that are present at relatively low concentrations (i.e., carotenes and cryptoxanthin in plasma; Table 2). Xanthophyll carotenoids also tended to vary a bit more independently in adipose tissue, where only around two-thirds of the correlations were statistically significant (Table 4).

Discussion

We investigated the carotenoid pigment profile of several internal and integumentary tissues in zebra finches, one of the best-studied animal models in the context of carotenoid mechanisms and functions (Blount 2004). Previous work of ours described the types and ratios of carotenoids present in certain internal tissues (e.g., plasma, liver, adipose; McGraw et al. 2002) and explored plasma carotenoid predictors of beak coloration (McGraw et al. 2003), but our aim in this study was to take a more quantitative approach by analyzing carotenoid pigment concentrations in more tissues to correlatively explore total-body carotenoid accumulation, allocation, and color acquisition, specifically as they may differ between the sexes.

Carotenoid profiles (i.e., types) were consistent among some but not all internal tissues. Plasma, liver, and adipose tissues were xanthophyll rich (>95% of total); this is a predicted occurrence in birds, which tend to be polar carotenoid accumulators (McGraw et al. 2003; but see Blount et al. 2002 and Faivre et al. 2003 for exceptions). Metabolically derived xanthophylls (anhydrolutein and dehydrolutein) co-occurred with dietary forms in these tissues as well. Retina, the fourth internal body tissue that we analyzed, differed substantially in carotenoid profile from these, however. Retina housed four different metabolic derivatives (galloxanthin and astaxanthin as major forms, epilutein and an unidentified carotene as minor components) and contained small amounts of only one other carotenoid (zeaxanthin) found elsewhere in the body. Retinal carotenoid profiles are thought to be quite conserved, at least among diurnal birds (Hart 2001*a*, 2001*b*), which perhaps underlies their consistent mechanisms and functions in the eye. Different types of avian retinal carotenoids apparently accumulate in oil droplets that are specific to the photoreceptor classes: droplets from shortwave-sensitive cones contain galloxanthin, those from medium wave–sensitive cones contain xanthophylls, and those from long wave–sensitive cones contain astaxanthin (Goldsmith et al. 1984). Such carotenoid specificity is thought to be linked to their unique light-absorbing roles as "cutoff" filters that further tune the sensitivities of each photoreceptor type (Vorobyev 2003; Cuthill 2006).

Very few studies to date (and only in chickens and quail; e.g., Stransky and Schulze 1977; Khachik et al. 2002) have qual-

Table 3: Intercorrelations among concentrations of carotenoid types within liver tissue

	Lutein	Zeaxanthin	Anhydrolutein	β-Cryptoxanthin	β-Carotene
Lutein		.94*	.97*	.90*	.79*
Zeaxanthin	.91*		.96*	.89*	.82*
Anhydrolutein	.71*	.72*		.89*	.81*
β-Cryptoxanthin	.45	.56*	.64*		.71*
β-Carotene	.69*	.73*	.59*	.78*	

Note. For more information on this table, see note in Table 2.

* $P < 0.05$.

Table 4: Intercorrelations among concentrations of carotenoid types within fat tissue

	Lutein Isomer	Dehydrolutein	Lutein	Zeaxanthin	Anhydrolutein	β-Cryptoxanthin
Lutein isomer		.97*	.33	.97*	.93*	.73*
Dehydrolutein	.87*		.21	.96*	.95*	.75*
Lutein	−.19	−.54		.41	.18	.13
Zeaxanthin	.83*	.58*	.30		.90*	.68*
Anhydrolutein	.91*	.69*	.05	.91*		.87*
β-Cryptoxanthin	.56*	.24	.13	.65*	.67*	

Note. For more information on this table, see note in Table 2.
* $P < 0.05$.

itatively or quantitatively investigated avian retinal carotenoids (Toomey and McGraw 2009). Instead, carotenoid identities are typically inferred from microspectrophotometric assessments of oil-droplet absorbance properties (Goldsmith and Butler 2005). As such, we still have much to learn about retinal carotenoid-accumulation patterns, especially in wild birds. Our work, however, lends some insights into the mechanistic uniqueness of avian retinal carotenoids. Perhaps because of the fact that they are housed in oil droplets, retinal carotenoids appear to be difficult to bleach from the eye (Meyer 1971; Wang et al. 2007) and do not seem to be as accessible for other uses. We found no indications of astaxanthin, galloxanthin, and so on, elsewhere inside the body, so it very much appears that the retina, like dead tissues (e.g., beak, leg), is an "endpoint" for carotenoids, as opposed to other internal body carotenoid sources—such as plasma, liver, and adipose tissue—that can be available for bodywide functions (although varying in temporal accessibility; Koutsos et al. 2003, but see Negro et al. 2001; see more below). Instead, the abundance of retinal carotenoids may relate to replacement rates (e.g., via dietary and other tissue sources, health factors) and perhaps other environmental variables, such as light exposure (as shown recently in an experiment with chickens; Hart et al. 2006; see below).

The two carotenoid-colored integumentary tissues in zebra finches—beak and legs—had altogether different pigment profiles. Orange to red beaks, as expected from previous pilot investigations (McGraw et al. 2002), were dominated by red ketocarotenoids, which we presume are made in the maturing beak tissue itself (McGraw 2004). Orange legs differed in that they contained only a small amount of one ketocarotenoid (canthaxanthin) and were pigmented mostly by dietary and metabolically derived xanthophylls. These unique carotenoid profiles, coupled with the facts that beak and leg color did not strongly positively covary in either sex and had different internal-tissue carotenoid predictors, suggest that these two tissues may reveal different information and perhaps then function as different signals (see below). Although a premium is placed on ketocarotenoid accumulation (all four red pigments in the beak, only one in the leg) for intense coloration in both tissues, the observation that leg carotenoid accumulation in males was linked particularly to fat carotenoid supplies (thought to be storage/reserve sources; Negro et al. 2001) suggests that it could represent a longer-term signal of carotenoid status (health/diet) than the beak, which is linked to currently circulating plasma levels and whose tissue is thought to be replaced at a more continuous/rapid rate in birds (e.g., white-crowned sparrows *Zonotrichia leucophrys*; King and Murphy 1990). Clearly, more work is needed to determine the function of zebra finch leg coloration; some have suggested that red leg-band color preferences by females is a product of their preferences for colorful legs in males (reviewed in Hill 2006), but to our knowledge no study has explicitly tested mate choice for natural leg color in zebra finches.

The sexes differed in carotenoid accumulation for all tissues tested, but unlike suggestions from previous work (McGraw et al. 2003) it was not the case that males always had higher levels than females in our study. Male zebra finches have beaks and legs that are more red-orange than those of females, and thus it was not surprising that males were found to have higher levels of red-orange ketocarotenoids in both tissues. However, liver and adipose tissue contained higher concentrations of carotenoids in females than did those same tissues in males. Given previous work showing no sex difference in carotenoid or food consumption in this species (McGraw et al. 2003), such tissue-

Table 5: Intercorrelations among concentrations of carotenoid types within retina tissue

	Galloxanthin	Epilutein	Astaxanthin	Zeaxanthin	?-Carotene
Galloxanthin		…	.71*	.82*	.27*
Epilutein	.86*		…	…	…
Astaxanthin	.79*	.74*		.60*	.23
Zeaxanthin	.92*	.95*	.79*		.72*
?-Carotene	.78*	.96*	.67*	.86*	

Note. Retinal ?-carotene and epilutein levels were undetectable in many females, so we could not report some of the correlations for them. For more information on this table, see note in Table 2.
* $P < 0.05$.

specific sex differences are consistent with functional differences in carotenoid allocation between males and females. Males appear to prioritize current carotenoid use by circulating high levels for delivery to integumentary tissues for coloration (and thus mate attraction). This circulating pool is also available for maintaining good health (via antioxidant or cell/gene regulatory mechanisms) and perhaps thereby maintaining signal honesty at this key time. Females in our study, in contrast, concentrated more carotenoids in storage tissues (adipose and liver), which may be important depots from which to draw for nutrient enrichment of egg yolks (Houston et al. 1995). Reproductive state may have been slightly accelerated in females (given that some laid eggs) compared with males here, but nonetheless this female allocation pattern makes sense because of the incredible investment that yolk carotenoids can represent (>50% of total-body carotenoids in some species; Bortolotti et al. 2003) and because dietary intake during the laying sequence cannot replenish carotenoid amounts lost to each egg (see Royle et al. 2003 for evidence of laying-order decreases in yolk carotenoid concentration).

Several other studies have found sex-specific carotenoid allocation patterns that are consistent with ours, although not comprehensively within one species as we found. In five other species with sexually dichromatic carotenoid coloration, males circulate more carotenoids through blood than females do (reviewed in McGraw et al. 2003; but see Bortolotti et al. 1996 for an example in a noncarotenoid-colored species); this pattern is season-specific in house finches, because only during molt do males accumulate more β-cryptoxanthin, the dietary carotenoid that is critical for red coloration (McGraw et al. 2006*c*; K. J. McGraw and M. B. Toomey, unpublished data). In Japanese quail, a species with no carotenoid ornamentation, females have higher plasma and tissue levels of carotenoids (Toyoda et al. 2002). However, in graylag geese (*Anser anser*)—a species with orange leg, eye ring, and beak coloration—males have higher fat carotenoid concentrations in winter than do females (Negro et al. 2001). More controlled comparative and experimental studies are now needed, perhaps at different times of year (when carotenoid allocation may shift according to breeding, color acquisition, health challenges, etc.), to further test this hypothesis about sex differences in temporal/functional allocation of carotenoids. In addition to reproductive mechanisms, hormonal (e.g., testosterone; Blas et al. 2006; McGraw et al. 2006*a*) and health factors underlying such sex-specific allocation patterns should also receive attention.

It was also interesting that, although there is no sex difference in retinal carotenoid concentration in wild house finches (Toomey and McGraw 2009), male zebra finches had higher retinal carotenoid levels than did females. Assuming that there is a functional basis to this, it is possible that higher levels in males may facilitate food-color discrimination (for pigment acquisition and sexual signaling; see McGraw 2006 for a review of selection of red food by songbirds) or aid in photoprotection (in the more active and thus more sun-exposed sex; Zann 1996). This stands counter to the prediction that females might be expected to have higher retinal carotenoid levels than males, in order to allow selection of potential mates based on orange-red color. However, it is important to note that males and females in our study were housed in different lighting environments (see "Methods"), which may have artificially created the sex differences that we observed, not only in retinal carotenoids but in other internal tissues as well. As Hart et al. (2006) found in an experiment with chickens, increased ambient light exposure can increase oil-droplet (carotenoid) pigmentation in retinas. Pale oil droplets (containing galloxanthin) were particularly affected by this treatment, and retinal galloxanthin concentration especially differed between the sexes in our study. Note that we would not expect these different housing conditions for males and females to necessarily confound sex differences observed in beak color and plasma carotenoid levels, however, as those patterns have been detected in previous studies when male and female zebra finches were housed under identical environmental conditions (McGraw et al. 2003; McGraw and Ardia 2005).

We examined carotenoid intercorrelations within and among tissue types for each sex to better understand (1) the pigment- and tissue-specific predictors of color development and (2) how animals accumulate levels of different carotenoids (e.g., xanthophylls vs. carotenes, dietary vs. metabolized forms). First, within each tissue type, we were curious how concentrations of different carotenoids covaried; such relationships may speak to general accumulation strategies ("more is better" if all are positively intercorrelated; McGraw and Gregory 2004) or to preferential mechanism-specific accumulation of particularly valuable pigments (for health, coloration, visual tuning, or photoprotection; McGraw et al. 2006*c*). In this study, we found consistent support for a generalized accumulation mechanism in internal tissues—when birds had more of one carotenoid type in any given tissue, they tended to accumulate more of all other types within that tissue. This suggests that there are few if any negative competitive interactions that may occur among carotenoid forms during uptake (van den Berg 1999). It also fails to support the notion that any particular substrate is rate limiting for the accumulation of metabolized forms; otherwise, we would have expected to see several negative correlations between dietary precursors and metabolized products within any tissue. We presume that such a phenomenon is controlled by a very general absorption, lipoprotein transport, and perhaps even a tissue-binding mechanism; our previous work on lipoprotein function in zebra finches supports this view, at least as it relates to plasma carotenoids (which all increased or decreased when lipoproteins were up- or down-regulated, respectively; McGraw and Parker 2006).

This generalized mechanism for carotenoid accumulation occurred in integumentary tissues as well. In fact, correlations among carotenoid types were strongest in beak and legs. However, such correlations translated into different predictors of color intensity in these two tissues. Birds with the most colorful beaks deposited more of all red ketocarotenoid types into this tissue. This is consistent with some bird species, where maximum coloration is generated by accumulating more of many different carotenoid types (e.g., American goldfinch *Carduelis*

tristis; McGraw and Gregory 2004), but it is different from a few select others that target specific carotenoids (e.g., the house finch) for developing maximal coloration (Inouye et al. 2001; McGraw et al. 2006*c*). Interestingly, this latter case appears to hold for the orange leg coloration of zebra finches: males who deposited more canthaxanthin into legs developed legs that were more orange. This, coupled with the lack of significant correlation between beak and leg hue within the sexes, further suggests that carotenoid-containing integumentary tissues in this species may carry different signal contents, with leg color being more indicative of a selective carotenoid-accumulation process (i.e., the ability to make or retain canthaxanthin over xanthophylls in leg tissue) and with beak color capturing more general physiological and metabolic processes associated with handling many carotenoid types in the blood and at the beak (i.e., the intercorrelations among beak hue, total-beak carotenoids, and total-plasma carotenoids).

As we further explored carotenoid intercorrelations, we found consistent positive correlations among the carotenoid concentrations in various tissues. In other words, if an individual of either sex accumulated high concentrations of carotenoids in liver, they also tended to have high levels in other internal and external tissues. Not all correlations were statistically significant, but several patterns are noteworthy.

First, plasma concentrations showed perhaps the most variable links to other tissue sources (e.g., three of the four negative correlations in Table 1 were for plasma). This suggests to us that carotenoid levels in a plasma sample, while easily and commonly assessed by researchers and certainly indicative of the current circulating pool available for functional allocation, can represent an integration that is quite complex (essentially, a running average) of several recent carotenoid-related processes: (1) carotenoid intake from food, (2) health/antioxidant depletion of carotenoids, (3) allocation to yolk (in laying females), (4) allocation to retina, (5) allocation to the integument, and (6) allocation to or retrieval from storage tissues (e.g., liver, fat). Biologists should keep in mind the variable influences of these dynamic processes (with season, breeding status, health, etc.) as they look to use plasma carotenoid concentration as a metric of general carotenoid status or of individual quality in their respective study species.

Second, plasma carotenoid concentrations in females were significantly negatively correlated with fat carotenoid concentrations, and we believe that this represents active carotenoid retrieval from tissue storage (Negro et al. 2001). As is typical for domesticated zebra finches, it is quite difficult to keep females from laying eggs (even in the absence of a mate, nest cup, nest material, etc.), and our technicians noted that some of the females were in fact laying eggs during the study. Hence, here we may have caught certain females in the process of retrieving carotenoids from fat for yolk deposition (i.e., those with low fat carotenoid levels had high levels in circulation).

Third, carotenoids in retina also showed variable correlations with specific tissue sources (significant for liver and beak in females; fat in males). That adipose carotenoids, which are so key to reproduction in females, would have a different relationship in males is yet another line of evidence for sex-specific carotenoid investment. Both retina and adipose tissue (in addition to leg tissue) seem to be sites at which carotenoids are sequestered for longer periods of time, so a mechanistic link between them in males makes sense. Moreover, that a female's beak color might be linked to her ability to see color (via retinal carotenoid action) raises interesting prospects for how carotenoid molecules themselves (in eyes and the integument) may be the common currency with which the coevolution of carotenoid-based color traits and preferences is maintained (Hill and McGraw 2004).

Acknowledgments

All procedures were approved by the Institutional Animal Care and Use Committee at Arizona State University (protocol 05-764R). We thank an anonymous reviewer for constructive comments on the manuscript. Funding for this work was provided by the School of Life Sciences and the College of Liberal Arts and Sciences at Arizona State University.

Literature Cited

Andersson S. and M. Prager. 2006. Quantifying colors. Pp. 41–89 in G.E. Hill and K.J. McGraw, eds. Bird Coloration. Vol. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, MA.

Blas J., L. Pérez-Rodríguez, G.R. Bortolotti, J. Viñuela, and T.A. Marchant. 2006. Testosterone increases bioavailability of carotenoids: insights into the honesty of sexual signaling. Proc Natl Acad Sci USA 103:18633–18637.

Blount J.D. 2004. Carotenoids and life-history evolution in animals. Arch Biochem Biophys 430:10–15.

Blount J.D., P.F. Surai, D.C. Houston, and A.P. Møller. 2002. Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. Funct Ecol 16:445–453.

Bortolotti G.R., J.J. Negro, P.F. Surai, and P. Prieto. 2003. Carotenoids in eggs and plasma of red-legged partridges: effects of diet and reproductive output. Physiol Biochem Zool 76: 367–374.

Bortolotti G.R., J.J. Negro, J.L. Tella, T.A. Marchant, and D.M. Bird. 1996. Sexual dichromatism in birds independent of diet, parasites and androgens. Proc R Soc B 263:1171–1176.

Burley N. and C.B. Coopersmith. 1987. Bill color preferences of zebra finches. Ethology 76:133–151.

Cuthill I.C. 2006. Color perception. Pp. 3–40 in G.E. Hill and K.J. McGraw, eds. Bird Coloration. Vol. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, MA.

Faivre B., A. Gregoire, M. Preault, F. Cezilly, and G. Sorci. 2003. Immune activation rapidly mirrored in a secondary sexual trait. Science 300:103.

Garamszegi L.Z. 2006. Comparing effect sizes across variables: generalizations without the need for Bonferroni correction. Behav Ecol 17:682–687.

Goldsmith T.H. and B.K. Butler. 2005. Color vision of the budgerigar (*Melopsittacus undulatus*): hue matches, tetrachromacy, and intensity discrimination. J Comp Physiol A 191:933–951.

Goldsmith T.H., J.S. Collins, and S. Licht. 1984. The cone oil droplets of avian retinas. Vision Res 24:1661–1671.

Goodwin T.W. 1980. Biochemistry of the Carotenoids. Vol. 1. Plants. Chapman & Hall, London.

Hargitai R., Z. Matus, G. Hegyi, G. Michl, G. Toth, and J. Torok. 2005. Antioxidants in the egg yolk of a wild passerine: differences between breeding seasons. Comp Biochem Physiol B 143:145–152.

Hart N.S. 2001*a*. Variations in cone photoreceptor abundance and the visual ecology of birds. J Comp Physiol A 187:685–698.

———. 2001*b*. The visual ecology of avian photoreceptors. Prog Retinal Eye Res 20:675–703.

Hart N.S., T.J. Lisney, and S.P. Collin. 2006. Cone photoreceptor oil droplet pigmentation is affected by ambient light intensity. J Exp Biol 209:4776–4787.

Hill G.E. 2006. Female mate choice for ornamental coloration. Pp. 137–200 in G.E. Hill and K.J. McGraw, eds. Bird Coloration. Vol. 2. Function and Evolution. Harvard University Press, Cambridge, MA.

Hill G.E. and K.J. McGraw. 2004. Correlated changes in male plumage coloration and female mate choice in cardueline finches. Anim Behav 67:27–35.

Houston D.C., D. Donnan, and P.J. Jones. 1995. The source of the nutrients required for egg production in zebra finches *Poephila guttata.* J Zool 235:469–483.

Inouye C.Y., G.E. Hill, R.D. Stradi, and R. Montgomerie. 2001. Carotenoid pigments in male house finch plumage in relation to age, subspecies, and ornamental coloration. Auk 118:900–915.

Khachik F., F.F. de Moura, D.Y. Zhao, C.P. Aebischer, and P.S. Bernstein. 2002. Transformations of selected carotenoids in plasma, liver, and ocular tissues of humans and in nonprimate animal models. Investig Ophthalmol Vis Sci 43:3383–3392.

King J.R. and M.E. Murphy. 1990. Estimates of the mass of structures other than plumage produced during molt by white-crowned sparrows. Condor 92:839–843.

Koutsos E.A., C.C. Calvert, and K.C. Klasing. 2003. The effect of an acute phase response on tissue carotenoid levels of growing chickens (*Gallus gallus domesticus*). Comp Biochem Physiol A 135:635–646.

McGraw K.J. 2004. Colorful songbirds metabolize carotenoids at the integument. J Avian Biol 35:471–476.

———. 2005. Interspecific variation in dietary carotenoid assimilation in birds: links to phylogeny and color ornamentation. Comp Biochem Physiol B 142:245–250.

———. 2006. The mechanics of carotenoid coloration in birds. Pp. 177–242 in G.E. Hill and K.J. McGraw, eds. Bird Coloration. Vol. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, MA.

———. 2007. Dietary mineral content influences melanin-based ornamental coloration. Behav Ecol 18:137–142.

McGraw K.J., E. Adkins-Regan, and R.S. Parker. 2002. Anhydrolutein in the zebra finch: a new, metabolically derived carotenoid in birds. Comp Biochem Physiol B 132:811–818.

———. 2005. Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. Naturwissenschaften 92:375–380.

McGraw K.J. and D.R. Ardia. 2003. Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. Am Nat 162:704–712.

———. 2005. Sex differences in carotenoid status and immune performance in zebra finches. Evol Ecol Res 7:251–262.

McGraw K.J., S.M. Correa, and E. Adkins-Regan. 2006*a*. Testosterone upregulates lipoprotein status to control sexual attractiveness in a colorful songbird. Behav Ecol Sociobiol 60: 117–122.

McGraw K.J., O.L. Crino, W. Medina-Jerez, and P.M. Nolan. 2006*b*. Effect of dietary carotenoid supplementation on food intake and immune function in a songbird with no carotenoid coloration. Ethology 112:1209–1216.

McGraw K.J. and A.J. Gregory. 2004. Carotenoid pigments in male American goldfinches: what is the optimal biochemical strategy for becoming colourful? Biol J Linn Soc 83:273–280.

McGraw K.J., A.J. Gregory, R.S. Parker, and E. Adkins-Regan. 2003. Diet, plasma carotenoids, and sexual coloration in the zebra finch (*Taeniopygia guttata*). Auk 120:400–410.

McGraw K.J., G.E. Hill, K.J. Navara, and R.S. Parker. 2004. Differential accumulation and pigmenting ability of dietary carotenoids in colorful finches. Physiol Biochem Zool 77: 484–491.

McGraw K.J., P.M. Nolan, and O.L. Crino. 2006*c*. Carotenoid accumulation strategies for becoming a colorful house finch: analyses of plasma and liver pigments in wild molting birds. Funct Ecol 20:678–688.

McGraw K.J. and R.S. Parker. 2006. A novel lipoprotein-mediated mechanism controlling sexual attractiveness in a colorful songbird. Physiol Behav 87:103–108.

McGraw K.J. and J.G. Schuetz. 2004. The evolution of carotenoid coloration in estrildid finches: a biochemical analysis. Comp Biochem Physiol B 139:45–51.

Meyer D.B. 1971. The effect of dietary carotenoid deprivation on avian retinal oil droplets. Ophthalmol Res 2:104–109.

Montgomerie R. 2006. Analyzing colors. Pp. 90–147 in G.E. Hill and K.J. McGraw, eds. Bird Coloration. Vol. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, MA.

Moran M.D. 2003. Arguments for rejecting the sequential Bonferroni in ecological studies. Oikos 102:403–405.

Moreno J. 1989. Strategies of mass change in breeding birds. Biol J Linn Soc 37:297–310.

Negro J.J., J. Figuerola, J. Garrido, and A.J. Green. 2001. Fat stores in birds: an overlooked sink for carotenoid pigments? Funct Ecol 15:297–303.

Peters A., K. Delhey, A.G. Denk, and B. Kempenaers. 2004.

Trade-offs between immune investment and sexual signaling in mallards. Am Nat 164:51–59.

Royle N.J., P.F. Surai, and I.R. Hartley. 2003. The effect of variation in dietary intake on maternal deposition of antioxidants in zebra finch eggs. Funct Ecol 17:472–481.

Speake B.K., F. Decrock, P.F. Surai, and R. Groscolas. 1999. Fatty acid composition of the adipose tissue and yolk lipids of a bird with a marine-based diet, the emperor penguin (*Aptenodytes forsteri*). Lipids 34:283–290.

Stransky H. and I. Schulze. 1977. Carotenoids in *Gallus domesticus*: comparative analysis of blood and retina of chickens and of egg-yolk. J Comp Physiol B 115:265–277.

Thomson L.R., Y. Toyoda, F.C. Delori, K.M. Garnett, Z.Y. Wong, C.R. Nichols, K.M. Cheng, N.E. Craft, and C.K. Dorey. 2002*a*. Long term dietary supplementation with zeaxanthin reduces photoreceptor death in light-damaged Japanese quail. Exp Eye Res 75:529–542.

Thomson L.R., Y. Toyoda, A. Langner, F.C. Delori, K.M. Garnett, N. Craft, C.R. Nichols, K.M. Cheng, and C.K. Dorey. 2002*b*. Elevated retinal zeaxanthin and prevention of light-induced photoreceptor cell death in quail. Investig Ophthalmol Vis Sci 43:3538–3549.

Toomey M.B. and K.J. McGraw. 2007. Modified saponification and HPLC methods for analyzing carotenoids from the retina of quail: implications for its use as a nonprimate model species. Investig Ophthalmol Vis Sci 48:3976–3982.

———. 2009. Seasonal, sexual, and quality related variation in retinal carotenoid accumulation in the house finch (*Carpodacus mexicanus*). Funct Ecol 23:321–329. doi:10.1111/j.1365-2435.2008.01498.x.

Toyoda Y., L.R. Thomson, A. Langner, N.E. Craft, K.M. Garnett, C.R. Nichols, K.M. Cheng, and C.K. Dorey. 2002. Effect of dietary zeaxanthin on tissue distribution of zeaxanthin and lutein in quail. Investig Ophthalmol Vis Sci 43:1210–1221.

van den Berg H. 1999. Carotenoid interactions. Nutr Rev 57: 1–10.

Vecchi M., E. Glinz, V. Meduna, and K. Schiedt. 1987. HPLC separation and determination of astacene, semiastacene, astaxanthin, and other keto-carotenoids. J High Resolut Chromatogr 10:348–351.

Vorobyev M. 2003. Coloured oil droplets enhance colour discrimination. Proc R Soc B 270:1255–1261.

Wald G. and H. Zussman. 1937. Carotenoids of the chicken retina. J Biol Chem 122:449–460.

Wang Y., S.L. Connor, W. Wang, E.J. Johnson, and W.E. Connor. 2007. The selective retention of lutein, meso-zeaxanthin and zeaxanthin in the retina of chicks fed a xanthophyll-free diet. Exp Eye Res 84:591–598.

Zann R.A. 1996. The Zebra Finch. Oxford University Press, Oxford.