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Colorful songbirds metabolize carotenoids at the integument

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For decades, carotenoids have attracted attention for their roles as vitamin-A precursors, antioxidants, and immunostimulants, but we still understand very little about the metabolic processes that accompany these compounds. Animals like birds use carotenoids to color their feathers and bare parts to become sexually attractive. They commonly metabolically derive their body colorants from dietary sources of carotenoids, but the sites of pigment metabolism remain unidentified. Here I test the hypothesis that songbirds manufacture their colorful feather and beak carotenoids directly at these tissues. I offer two lines of evidence to support this idea: (1) in a study of 11 colorful species from three passerine families, metabolically derived feather and beak carotenoids were found neither in the liver (a purported site of carotenoid metabolism), nor in the bloodstream (the means by which metabolites would be transported to colorful tissues from anywhere else in the body) at the time when pigments were being deposited into keratinized tissue, and (2) in a more detailed study of pigmentation in the American goldfinch *Carduelis tristis*, carotenoids sampled from the lipid fractions of maturing feather follicles yielded a mix of dietary and synthetic carotenoids, suggesting that this is the metabolically active site for feather-pigment production. This fresh perspective on carotenoid metabolism in animals should aid our efforts to characterize the responsible enzymes and to better understand the localized biological functions of these pigments.

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Carotenoids are a class of over 600 natural compounds that serve diverse biological functions, including light harvesting during photosynthesis, free radical scavenging, and immunostimulation (Vershinin 1999). Animals like birds and fishes also use these pigments to color portions of their body as a means of attracting mates (Olson and Owens 1998, Hill 1999). These organisms acquire carotenoid colorants from the diet, so it has long been thought that individuals advertise their foraging ability, nutritional state, and health with their bright colors (Endler 1983, Hill 1992, Lozano 1994).

Not all carotenoids that we see in animals are the same ones present in food, however. Many species

metabolize ingested pigments into alternate, more oxidized forms that are deposited into the integument (Schiedt 1989, 1998, Brush 1990). Colorful songbirds, for example, manufacture yellow canary xanthophylls and a host of red ketocarotenoids that appear in feathers, but are absent from the diet (Stradi 1998, McGraw et al. 2001). In these species, it has been argued that metabolic transformations of carotenoids are costly, energy-demanding processes that only the highest-quality individuals can complete to become the most colorful (Hill 1996, 2000).

Unfortunately, we lack critical tests of this hypothesis because we still have only a very basic understanding of

carotenoid metabolism in birds (McGraw et al. 2003a). To date, no enzymes responsible for these carotenoid conversions have been characterized. Remarkably, it is not even known where these integumentary pigments are transformed in the body. It is well documented in humans and other animals that the cleavage of β -carotene into vitamin A occurs in the liver and small intestine (Wyss et al. 2001). Similarly, some have suspected that the liver is the primary site at which the metabolically derived carotenoids of feathers and bare parts are made in colorful birds (Schiedt et al. 1985, Torrissen et al. 1989, Brush 1990). However, there are reports in the literature that suggest otherwise. Inouye (1999) analyzed liver tissue in wild male house finches *Carpodacus mexicanus* at the time when they were growing their colorful, carotenoid-containing feathers, but found no traces of metabolically derived plumage carotenoids. Although he advocated the liver as the 'most logical site for metabolic processing', Brush (1990) noted that the liver does not usually yield large quantities of carotenoids in colorful birds.

A deeper search of the literature suggests in fact that these colorants may be made in peripheral colorful tissues themselves. Kritzler (1943) was one of the first to suppose that the feather germ may be the site for carotenoid synthesis, since liver and adipose tissue in canaries *Serinus canaria* contained only dietary carotenoids and lacked the yellow plumage pigment, canary xanthophyll. More recently, the observation was made that astaxanthin is absent from the blood of flamingos and may be made in feather follicles, or fluids of pen feathers (Brush 1978) and in tarsal skin (Fox et al. 1967). Stradi (1998) and Inouye (1999) also hinted that the oxidation of ingested carotenoids probably occurs in the feather follicle.

I used two approaches to investigate whether songbirds form their integumentary carotenoids directly at colorful feathers and bare parts. First, I examined the carotenoid content of liver and blood for 11 passerine species at the time when they were developing and/or displaying metabolically derived carotenoid coloration. Since carotenoids are delivered to peripheral tissues in the body through the bloodstream, I could determine whether metabolically derived integumentary pigments were being locally produced if they were absent from liver and blood. To test this idea further, I characterized the carotenoid content of feather follicles in one of these species, the American goldfinch *Carduelis tristis*. Lipids accumulate within feather germs as lipoidal droplets (Lucas and Stettenheim 1972, Menon and Menon 2000), and if carotenoids are in fact being formed at the maturing feather, I expected to find either synthetic products alone or a mix of dietary and synthetic carotenoids in the droplets of growing yellow feathers from wild male goldfinches.

Methods

Study species

I studied serum, liver, and integumentary carotenoids in the following songbird species that span 8 genera and 3 families: zebra finch *Taeniopygia guttata*, common waxbill *Estrilda astrild*, black-rumped waxbill *Estrilda troglodytes*, zebra waxbill *Amandava subflava*, red avadavat *Amandava amandava*, diamond firetail *Stagonopleura guttata*, star finch *Neochmia ruficauda*, pin-tailed whydah *Vidua macroura*, greenfinch *Carduelis chloris*, house finch, and American goldfinch (Table 1). Members of the first eight species all develop a rich red beak (Goodwin 1982), along with variable carotenoid-based plumage colors (with the exception of the whydah) that were not considered here (because feather molt was not predictable in these captive birds). House finches display red, orange, and yellow carotenoid-based plumage coloration on the crown, breast, and rump (Hill 2002). Greenfinches (Saks et al. 2003) and American goldfinches (Middleton 1993) acquire lemon-yellow plumage across much of their body.

Sources of animals

Zebra waxbills, common waxbills, and pin-tailed whydahs were captured from the wild in South Africa and housed in captivity for at least 11 months prior to sampling (see McGraw and Schuetz 2004 for more details). Star finches, black-rumped waxbills, red avadavat, and diamond firetails were obtained from local pet stores or aviculturists (McGraw and Schuetz 2004). Zebra finches were raised in captivity in our indoor finch colony (McGraw et al. 2002). Six molting male house finches were trapped from the wild in the fall of 2001 in Lee County, Alabama, USA (see Hill 2002 for more details). Six molting male greenfinches were captured in mistnets in October 2002 at the Vaibla bird station in central Estonia (see Saks et al. 2003 for more details). Four molting American goldfinches were trapped from the wild in Tompkins County, New York, USA in the spring of 2001 (see McGraw and Gregory 2004 for more details).

Blood and tissue sampling

I characterized carotenoid profiles from blood, liver, and integument of all study species by combining data from the published literature with my own biochemical analyses. This allowed me to maximize taxonomic coverage and to ensure complete datasets for species in which carotenoids had been described from only one or two of these sources. Data for liver and plasma carotenoids in the estrildid finches were obtained from

Table 1. A list of carotenoids found in the blood, liver, and integument (feathers or beak) in eleven songbird species (Class Aves, Order Passeriformes) representing three families. Carotenoid types are assigned numbers in the table that correspond to the names listed below in the key. Note that, with the exception of the house finch (which deposits dietary carotenoids in feathers in addition to synthetic ones found nowhere else in the body), carotenoids present in the feathers or beak exist only in these tissues, and not in blood or liver. Sample sizes (number of birds studied) are given in parentheses for new data presented in this paper.

Family	Species	Blood	Liver	Integument	Other data sources	
Estrildidae	Zebra finch <i>Taeniopygia guttata</i>	1,2,3,7,8	1,2,3,7,8	11,12,13,14 (5)-beak	McGraw et al. 2002	
	Common waxbill <i>Estrilda astrild</i>	1,2,3,7,8 (3)	1,2,3,7,8 (3)	11,12,13,14 (3)-beak ^a	McGraw et al. 2002	
	Black-rumped waxbill <i>Estrilda troglodytes</i>	no data	1,2,3,7,8 (1)	11,12,13,14 (1)-beak ^a	McGraw and Schuetz 2004	
	Zebra waxbill <i>Amandava subflava</i>	1,2,3,7,8 (2)	1,2,3,7,8 (3)	11,12,13,14 (3)-beak ^a	McGraw et al. 2002	
	Red avadavat <i>Amandava amandava</i>	no data	1,2,3,7,8 (2)	11,12,13,14 (2)-beak ^a	McGraw and Schuetz 2004	
	Diamond firetail <i>Stagonopleura guttata</i>	1,2 (2)	1,2 (1)	11,12,13,14 (1)-beak ^a	McGraw and Schuetz 2004	
	Star finch <i>Neochmia ruficauda</i>	no data	1,2 (1)	11,12,13,14 (1)-beak ^a	McGraw and Schuetz 2004	
	Viduidae	Pin-tailed whydah <i>Vidua macroura</i>	1,2 (2)	1,2 (3)	11,12,13,14 (1)-beak ^a	none
		Fringillidae	House finch <i>Carpodacus mexicanus</i>	1,2,3,4,5,6 (6)	1,2	1,2,3,4,8,9,10,11,12,13,14,15,16,17-feathers
Greenfinch <i>Carduelis chloris</i>	1,2 (3)		1,2 (6)	9,10-feathers	Saks et al. 2003	
American goldfinch <i>Carduelis tristis</i>	1,2 (4)		1,2 (4)	9,10-feathers	McGraw et al. 2001	

^a Beak pigments are hypothetical, based on spectral-absorbance comparisons with zebra finch beak pigments (McGraw et al. 2002, R. Stradi, unpubl. data).

Key to carotenoids: 1 = lutein, 2 = zeaxanthin, 3 = β -cryptoxanthin, 4 = β -carotene, 5 = rubixanthin, 6 = gazaniaxanthin, 7 = 2', 3'-anhydrolutein, 8 = 3'-dehydrolutein, 9 = canary xanthophyll A, 10 = canary xanthophyll B, 11 = astaxanthin, 12 = α -doradexanthin, 13 = adonirubin, 14 = canthaxanthin, 15 = 3-hydroxy-echinenone, 16 = echinenone, 17 = 4-oxo-rubixanthin.

McGraw and Schuetz (2004), see also McGraw et al. (2002) for zebra finches. Data for plumage carotenoids in house finches, greenfinches, and goldfinches were obtained from Inouye et al. (2001), Saks et al. (2003), and McGraw et al. (2001), respectively. See Inouye (1999) for data on liver carotenoids in house finches.

I collected 80–120 μ L of whole blood through the alar vein from 5 of the captive-housed species and from wild goldfinches. See H \ddot{o} rak et al. (2004) and Hill (2002) for details on blood sampling in greenfinches and house finches, respectively. In goldfinches, I plucked newly growing feather primordia that had not yet melanized at the base and sampled the yellow lipid droplet that oozed from the shaft (*sensu* McGraw et al. 2003b). All birds except house finches were then briskly euthanized under a stream of carbon dioxide. With a razor, I cut off thin slices (ca 1 mg) of surface beak tissue. Birds were then dissected and their livers removed. All samples were stored in Eppendorf tubes at -80° C for later analysis.

Pigment extraction and analysis

Solvent extractions and high-performance liquid chromatography techniques for analyzing plasma and liver carotenoids follow McGraw et al. (2002). Extraction and HPLC procedures for feathers follow McGraw et al. (2003a) and for lipid droplets follow McGraw et al. (2003b). Beak carotenoids could not be identified with

our HPLC system because of pigment esterification, so I used absorbance spectrophotometry to compare the spectral profiles for estrildid and viduid finches to that previously determined for the suite of four keto-carotenoids in zebra finch beaks (McGraw et al. 2002, R. Stradi unpublished data). This method only provides a gross characterization of the overall light-absorbance properties of all pigments in the beak, but because: (1) absorbance characteristics in zebra and other estrildid finches were identical, and (2) red beak carotenoids differ from yellow pigments found elsewhere in the body of zebra finches (McGraw et al. 2002), I am confident that red carotenoids are unique to the beak in other estrildids as well (see Results).

Results

A variety of hydroxy- and keto-carotenoids were characterized from the plasma, liver, feathers, and beak of these 11 species. Common dietary carotenoids such as lutein and zeaxanthin were found in all plasma and liver samples (Table 1). Diet pigments like β -cryptoxanthin and β -carotene were less prevalent, with the former present in three of the estrildids (both liver and plasma) and the house finch (plasma only) and the latter found only in house finch plasma. Rubixanthin and gazaniaxanthin were also described from plasma (but not liver) only in house finches (Table 1). Among the dietary

carotenoids, only lutein and zeaxanthin were found the integument and only from house finches (Table 1).

Metabolically derived carotenoids were present in the colorful feathers or beak of all species. In five of the estrildid finches, two yellow metabolic derivatives – 2', 3'-anhydrolutein and 3'-dehydrolutein – were present in plasma and liver, but absent from red beaks (Table 1). Critical to the hypothesis regarding the site of carotenoid metabolism for integumentary pigments, the synthetic carotenoids found in all feathers and beaks analyzed were absent from plasma and liver in every species (Table 1). Zebra finches, and probably their estrildid and viduid relatives, use a suite of red ketocarotenoids, including astaxanthin and α -doradexanthin, found only in the beak, greenfinches and goldfinches deposit unique canary xanthophylls into feathers, and house finches make a range of yellow (e.g. canary xanthophylls) and red pigments (e.g. 3-hydroxy-echinenone, astaxanthin) that are restricted to colorful plumage (Table 1).

When I analyzed lipid droplets from maturing goldfinch feather follicles, I found a mixture of both dietary and synthetic carotenoids. Four pigments were present in all; dietary sources of lutein ($29 \pm 3.5\%$ of total, mean \pm SE) and zeaxanthin ($10.5 \pm 1.7\%$) were accompanied by the two feather pigments-canary xanthophyll A ($29.3 \pm 3.1\%$) and B ($31.5 \pm 3.2\%$). Dietary carotenoids comprised nearly 40% of total carotenoids in these fractions.

Discussion

I considered the anatomical origin of carotenoid metabolites present in the colorful integumentary tissues of several songbird species. Despite decades of work on the types and amounts of dietary and tissue carotenoids in carotenoid-colored birds (Brush 1981, Goodwin 1984), no study has demonstrated the site in the body at which these external colorants are formed. In eleven species from three passerine families, I found that the metabolically derived carotenoids present in feathers and beaks were absent both from liver – a hypothesized site of carotenoid metabolism by some (Brush 1990, Inouye 1999) – and the bloodstream – the vehicle that circulates lipids to peripheral body tissues (Erdman et al. 1993, Parker 1996). This implies that integumentary carotenoids are being formed locally at these colorful tissues. In fact, I was able to capture the bioconversion process in action, as biochemical analyses confirmed that lipid droplets from newly growing carotenoid-enriched American goldfinch feather shafts contained a mixture of dietary and metabolically derived carotenoids.

Thus, the integument should now be considered an important metabolically active site for carotenoids in birds, as it is in other animals. In mice and humans, the central cleavage of β -carotene into vitamin A is known to occur in skin melanocytes and keratinocytes

(Zouboulis 2000, Andersson et al. 2001, Redmond et al. 2001). Fishes appear to be uniquely capable of reducing ingested oxocarotenoids (like astaxanthin and canthaxanthin) into hydroxy- and unsubstituted-carotenoids (like zeaxanthin and β -carotene) in skin (Kitahara 1983, Metusalach et al. 1996). More relevant to the oxidative transformations in birds, certain fish (e.g. goldfish, *Carassius auratus*; fancy carp, *Cyprinus carpio*) also can oxidize zeaxanthin into astaxanthin in skin (Hata and Hata 1972, 1976). Thus, despite being a previously overlooked metabolically active tissue in birds, the integument seems to be a common site for carotenoid synthesis across animals.

The integument is by no means the only tissue known to have metabolic activity toward carotenoids, however. In addition to the aforementioned hepatic and duodenal conversions of pro-vitamin-A carotenoids in animals ranging from chickens to mice to humans, the enzymes that drive these reactions (see more below) have also been reported from the kidney and lungs of chickens and the kidney and testes of mice (Redmond et al. 2001, Lakshman 2004, Wyss 2004). Also, the retinas of mammals (e.g. mice, cows, humans, and monkeys) and chickens metabolize both β -carotene into vitamin A (Bhatti et al. 2003) and dietary xanthophylls (e.g. lutein, zeaxanthin) into their more oxidized derivatives (e.g. anhydrolutein, dehydrolutein; Schiedt et al. 1991, Khachik et al. 2002). The liver in several of the colorful birds used in this study completes these very same xanthophyll modifications (e.g. 5 of the estrildid finches; see McGraw et al. 2002). However, the important observation here is that these liver derivatives do not appear in feathers and bare parts, but rather these birds use carotenoids delivered through the bloodstream to generate their metabolically derived body colorants right at the integument. It will be exciting now to see how many other avian species restrict metabolism of display carotenoids to epidermal tissues.

Last, I consider the evolutionary significance of local production of integumentary carotenoids in colorful birds. As indicated above, the incorporation of copious amounts of metabolically derived carotenoids into the beak and feathers serves the valuable function of attracting mates in at least four of the songbirds studied here (house finch, Hill 2002; greenfinch, Eley 1991; American goldfinch, Johnson et al. 1993; zebra finch, Burley and Coopersmith 1987). If these are in fact expensive pigments to manufacture, it would make sense that they are handled efficiently, being produced specifically at the site of use and not risking poor delivery or free-radical-damage while en route from distant tissues. Now that we have target metabolically active tissues, we can begin to search for candidate enzymes that complete these transformations (e.g. 4-oxygenase, Stradi et al. 1996; mixed function oxidase, Hudon 1994) and perhaps use *in-vitro* feather-germ models to determine how

energy demanding these reactions really are. Still, we should not overlook the fact that these derived pigments are more oxidized than their dietary precursors and thus may be better cellular antioxidants (Mortensen and Skibsted 1997). This same molecular property may also make them more photostable and less susceptible to photobleaching as they are contained in the integument over long periods of time.

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