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Lutein-based plumage coloration in songbirds is a consequence of selective pigment incorporation into feathers

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Abstract

Many birds obtain colorful carotenoid pigments from the diet and deposit them into growing tissues to develop extravagant red, orange or yellow sexual ornaments. In these instances, it is often unclear whether all dietary pigments are used as integumentary colorants or whether certain carotenoids are preferentially excluded or incorporated into tissues. We examined the carotenoid profiles of three New World passerines that display yellow plumage coloration—the yellow warbler (*Dendroica petechia*), common yellowthroat (*Geothlypis trichas*) and evening grosbeak (*Coccothraustes vespertinus*). Using high-performance liquid chromatography, we found that all species used only one carotenoid—lutein—to color their plumage yellow. Analyses of blood carotenoids (which document those pigments taken up from the diet) in two of the species, however, revealed the presence of two dietary xanthophylls—lutein and zeaxanthin—that commonly co-occur in plants and animals. These findings demonstrate post-absorptive selectivity of carotenoid deposition in bird feathers. To learn more about the site of pigment discrimination, we also analyzed the carotenoid composition of lipid fractions from the follicles of immature yellow-pigmented feathers in *G. trichas* and *D. petechia* and again detected both lutein and zeaxanthin. This suggests that selective lutein incorporation in feathers is under local control at the maturing feather follicle.

Keywords: Carotenoids; *Coccothraustes vespertinus*; Common yellowthroat; *Dendroica petechia*; Evening grosbeak; *Geothlypis trichas*; HPLC; Physiological discrimination; Yellow warbler; Zeaxanthin

1. Introduction

Many animals, particularly birds and fishes, use carotenoid pigments to develop striking red, orange and yellow patches of color that are used as signals of quality to attract mates (reviewed in Olson and Owens, 1998; Hill, 1999; Møller et al., 2000). To develop their sexual colors, animals must acquire these pigments from the diet and deliver them to peripheral tissues such as feathers and skin for pigmentation (reviewed in McGraw and Hill, 2001). The physiological underpinnings of carotenoid coloration have been of recent interest as potentially costly means of exaggerating colorful sexual traits (Hill, 1996, 2000; McGraw et al., in press).

For most carotenoid-pigmented species, however, the physiological processes that govern the expression of pigment-based ornaments are not

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well defined. While the actual chemical compounds used to color feathers and bare parts have now been described for several fishes (Torrissen et al., 1989; Scheidt, 1998) and songbirds (Stradi, 1998), it is rare that we also know the full complement of carotenoids that these animals acquire from the diet or how these animals physiologically apportion the available pigments for color display. It is conceivable that animals follow very specific biochemical strategies for becoming colorful, selectively accumulating and displaying only certain pigments, while failing to utilize others or perhaps saving them for different physiological functions (e.g. immunomodulation, vitamin synthesis). Only with detailed accounts of the carotenoids present in the diet and body fluids and tissues can we begin to understand the biochemical specificity of these pigmentation systems.

In this study, we used high-performance liquid chromatography (HPLC) to characterize the carotenoid pigments present in three colorful songbird species (Aves: Passeriformes)—the yellow warbler (*Dendroica petechia*), the common yellowthroat (*Geothlypis trichas*) and the evening grosbeak (*Coccothraustes vespertinus*)—so that we could follow the fate of carotenoids through the body to feathers. These species are united by the colorful yellow plumage that they display throughout the year and regrow each autumn. In all of the species, there is some evidence suggesting that this presumed carotenoid-based plumage coloration serves a sexual function. Male warblers, yellowthroats and grosbeaks develop more colorful yellow plumage than do females of their species (K.J.M., pers. obs.), and more colorful male yellowthroats acquire more extra-pair mates than less colorful individuals (C. Freeman-Gallant, unpublished data).

First, we obtained yellow breast feathers from all species and determined their carotenoid composition. We were also able to capture yellow warblers and yellowthroats from the wild at the time they were growing their colorful feathers. In these individuals, we obtained a recent record of those carotenoids taken up from the diet by drawing a blood sample. We then compared their blood-carotenoid profile with that of freshly plucked yellow feathers, to determine which circulated pigments were being used as plumage colorants. Last, we found feathers on some molting individuals that were at a very early stage of growth—at a time when carotenoids were still being deposited into feathers and only a portion of their yellow-tipped contour feathers had emerged from the sheath. By collecting these 'primordial' feathers, we were able to analyze the carotenoids contained within lipid droplets that accumulate in cells of the feather blastema prior to deposition (Desselberger, 1930; Lucas and Stettenheim, 1972; Menon and Menon, 2000), to document those pigments that are delivered directly to the feather follicle for pigmentation. This allowed us to determine whether feather follicles could exert local control over pigment deposition by discriminating among several different carotenoids that were transported to the site through the blood.

2. Methods

2.1. Collection of samples

During the breeding season of 2002, M.D.B. collected yellow breast feathers from each of three wild-caught male and female yellow warblers in Pennsylvania. These feathers were stored in sealed envelopes in the dark until they were analyzed in October 2002. On 14 August 2002, K.J.M. captured a molting male yellow warbler and a molting male common yellowthroat in Ithaca, New York. From each individual, we plucked 5 yellow breast feathers and collected 20–40 μl of whole blood. Blood was centrifuged and the plasma saved for analysis. From these birds, we also collected 10 still-growing ('primordial') contour feathers that were emerging from their sheaths. All of these samples were analyzed later that day. On 14 September 2002, P. Dunn collected yellow breast feathers and blood from three wild-caught molting yellowthroats (2 males, 1 female) in Saukville, Wisconsin. Plasma and feathers were stored at −80 °C and analyzed in mid-October 2002. Last, we obtained breast feathers from 10 evening grosbeaks as donations from the Auburn University Natural History Museum (4 males, 4 females) and the Museum of Vertebrates at Cornell University (1 male, 1 female). These specimens ranged in age from 4 to 33 years.

2.2. Carotenoid analyses

2.2.1. Fully grown feathers

Feathers were first washed in ethanol and hexane (sequentially) to remove surface lipids. We then blotted the feathers dry on filter paper,

trimmed off the yellow-pigmented barbules and added these to 1-ml acidified pyridine in a 9-ml glass tube (*sensu* Hudon and Brush, 1992; McGraw et al., 2002a). We filled the headspace of the tube with argon and held the solution at 95 °C for 3 h. After cooling to room temperature, we added 1-ml water, vortexed the mix and then added 1-ml *tert*-butyl methyl ether. We shook the tube for 2 min and centrifuged at 3000 RPM for 5 min. The upper, colored phase (containing the carotenoids) was transferred to a 1.5-ml HPLC vial and evaporated to dryness under a stream of nitrogen. We resuspended the carotenoids in 200-μl HPLC mobile phase (methanol:acetonitrile, 1:1, v/v) prior to analysis.

We injected 50 μl of each sample into a Waters™ 717plus Autosampler HPLC (Millipore Corp., Bedford, MA) fitted with a Develosil RPA-queous RP-30 column (250×4.6 mm ID; Nomura Chemical Co. Ltd, Japan). We used an isocratic system (HP 1050 Series Isocratic Pump) at a constant flow rate of 1 ml/min for 45 min. Data were collected from 250 to 600 nm using a Waters™ 996 photodiode array detector (Waters Chromatography, Milford, MA). Feather pigments were identified by comparing their retention times (t_R) and absorption properties (λ_{max} values) with authentic reference carotenoids donated by Roche Vitamins Inc. (Parsippany, NJ) and Dr Riccardo Stradi (University of Milan, Italy).

2.2.2. Plasma

In a 1.5-ml Eppendorf tube, we added 100-μl ethanol to 10-μl thawed plasma. We vortexed the tube for 5 s and then added 100-μl *tert*-butyl methyl ether. After vortexing again for 5 s, we centrifuged the tube for 4 min in an Eppendorf centrifuge (model 5414). The supernatant was removed, transferred to a clean HPLC vial and evaporated to dryness under nitrogen. HPLC analytical procedures follow those given above.

2.2.3. Primordial feathers

Freshly plucked immature feathers often oozed a droplet of yellow liquid out the base of the shaft. Carotenoids are known to accumulate in these lipoidal droplets before they are embedded into feathers during the final stages of keratinization (Desselberger, 1930; Lucas and Stettenheim, 1972; Menon and Menon, 2000). To analyze carotenoids in this lipid fraction, we added 100-μl ethanol to 10-μl droplet in an Eppendorf tube and vortexed for 10 s. Then we added 100-μl *tert*-butyl methyl ether before vortexing for another 10 s, centrifuging, removing the supernatant and evaporating the solvent in a fresh HPLC tube (as above). To be sure that we acquired all of the not-yet-keratinized carotenoids from the feather primordia, we also dissected the sheath away from the immature feather and rinsed out remaining lipids with 100-μl ethanol and 100-μl *tert*-butyl methyl ether (recall that feather carotenoids are bound tightly in keratin and cannot be removed using simple organic-solvent-based techniques such as this one). These two fractions (droplet + rinse) containing all undeposited pigments were pooled for HPLC analysis.

3. Results

3.1. Carotenoids in yellow feathers

HPLC chromatograms showed that the yellow plumage from all three species contained the same carotenoid profile (Fig. 1). One peak, identified as all-*trans* lutein ($t_R = 18.7$ min, $\lambda_{max} = 448$ and 476 nm), comprised over 60% of all eluted products. The remaining 40% of signals consisted of four peaks that matched the retention times and absorbance properties of a series of *cis* lutein isomers (Fig. 1). On the basis of tests using pure (all-*E*), crystalline lutein, we found that these *Z* isomers are formed as artifacts during the thermochemical extraction procedure (Mays et al., in press).

3.2. Carotenoids in plasma

Although only one carotenoid was detected in feathers, we identified two main xanthophylls in the plasma of molting male yellowthroats and yellow warblers: lutein and zeaxanthin ($t_R = 21.0$ min, $\lambda_{max} = 453$ and 481 nm) (Table 1). Lutein was still the predominant pigment, occurring at greater than 85% of total in all samples (Table 1). In addition to these polar xanthophylls, small quantities of two non-polar pigments—β-cryptoxanthin (2–3% of total) and β-carotene ($\approx$1% of total)—were also present (see McGraw et al., 2002b for methods).

3.3. Carotenoids in lipid fractions from primordial feathers

Because these songbirds appeared to be preferentially accumulating lutein as a plumage pigment,

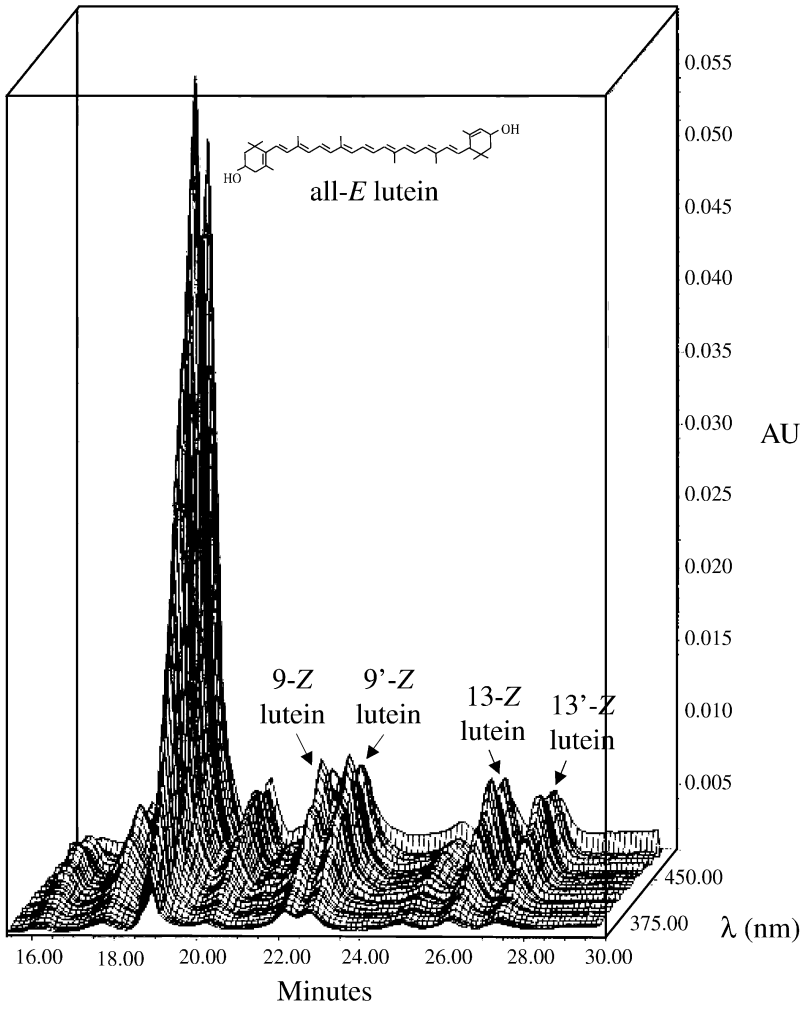

Fig. 1. Representative HPLC chromatogram of yellow feathers from an adult male common yellowthroat. We found all-*trans* lutein in these feathers, plus *cis* isomers that were formed during the thermochemical extraction process (Mays et al., in press; see Methods for additional details of the analytical HPLC procedure). Chromatograms for the other two yellow-colored bird species studied here were identical to this one.

Table 1
Carotenoid composition (% of total ± S.E.) of fully formed feathers, blood and unkeratinized lipid fractions from maturing feather follicles in four passerines that display yellow plumage coloration

Species	Site	*n*	Lutein (%)	Zeaxanthin (%)
Common yellowthroat	Feather	5	100	0
	Blood	4	89 ± 2	9 ± 2
	Follicle	1	85	14
Yellow warbler	Feather	7	100	0
	Blood	1	85	12
	Follicle	1	86	13
Evening grosbeak	Feather	10	100	0
Yellow-breasted chat	Feather	21	100	0

Data from yellow-breasted chats are taken from Mays et al. (in press).

we were interested in examining the anatomical site of pigment discrimination. Since circulating levels in blood should reach the maturing feather, we decided to test the carotenoid content of the lipid fractions not yet keratinized into growing feathers. Extracts from primordial feathers in yellowthroats and yellow warblers yielded notable levels of both lutein and zeaxanthin, closely matching those found in blood (Table 1; it should be noted that the yellowthroat from which primordial feathers were plucked showed a lutein:zeaxanthin ratio of 86:14 in plasma). Subsequent tests of the keratinized, yellow feather portions from these primordia (carotenoids extracted with heated pyridine) still yielded lutein only.

4. Discussion

For decades, physiological modes of carotenoid discrimination have been of interest to biochemists, particularly those studying human nutrition and the means by which carotenoids with pro-vitamin A activity (e.g. β-carotene) are assimilated from dietary sources (Erdman et al., 1993; Parker, 1996; Furr and Clark, 1997). Within the last few years, there has been a parallel attraction to the process of carotenoid utilization in birds, but as it relates to the allocation of carotenoids to the nutritive egg-yolk (Surai et al., 1998, 2001a; Blount et al., 2002) and subsequently to the tissues of developing embryos (Surai and Speake, 1998; Surai et al., 2001b). With the emerging interest in the mechanisms that control the expression of colorful carotenoid-derived feather colors, we set out to study the extent to which pools of circulating carotenoids were selectively incorporated into growing feathers.

We investigated the carotenoid profiles of three yellow-colored passerines to learn more about the degree to which specific carotenoid pigments were physiologically favored or disfavored as plumage colorants. These birds all used lutein as the lone pigment in yellow feathers. This is also true of a fourth songbird species with yellow plumage—the yellow-breasted chat (*Icteria virens*; Mays et al., in press). The presence of only a single carotenoid pigment in feathers is rare among birds. Most species use a mixture of dietary compounds or metabolic derivatives (e.g. canary xanthophylls, 4-oxo-carotenoids) to color their feathers (Stradi, 1998); there are only a few instances in which a single red pigment confers color on feathers (e.g. 3-hydroxy-echinenone in the long-tailed tit, *Aegithalos caudatus*; Stradi, 1998). Moreover, in species that use lutein as a feather colorant, its close molecular relative, zeaxanthin, or a zeaxanthin-derivative such as dehydrolutein, almost always co-exist (Stradi, 1998). This finding begged the question of whether or not lutein was the sole pigment available to these birds for incorporation into feathers.

To address this issue, we sampled blood from yellowthroats and yellow warblers at the time of feather growth and determined the carotenoid content of plasma. We detected a series of four carotenoids in the blood of these molting birds. Two xanthophylls, lutein and zeaxanthin, were predominant, making up over 95% of all carotenoids in blood. This is a common feature of songbirds, including many finches (e.g. American goldfinch, *Carduelis tristis*; K.J.M., unpublised data) and sparrows (e.g. northern cardinal, *Cardinalis cardinalis*; K.J.M., unpublished data), although lutein was comparatively more abundant than zeaxanthin than has been observed previously. This may be a reflection of the mainly insect-based foods that the warblers in this study consume, as opposed to the typical seed and fruit diets of finches and sparrows (Goodwin, 1980, 1984). In addition to these polar xanthophylls, we also isolated very small quantities of two non-polar carotenoids—β-carotene and β-cryptoxanthin. It is unclear if these low concentrations were due to low levels in the diet, inefficient uptake (see more below) or rapid clearance from the system via hepatic and intestinal conversion to vitamin A (Wyss et al., 2001).

Nevertheless, the absence of zeaxanthin, β-carotene and β-cryptoxanthin in feathers suggests that, at some level, these birds are discriminating among potential plumage pigments in blood and selectively accumulating lutein over all others. The failure of these warblers to use β-carotene and β-cryptoxanthin is not all that surprising, given the widely held view that birds, reptiles and fishes, unlike humans, selectively accumulate polar hydroxy- and ketocarotenoids over non-polar carotenes (Schiedt, 1989; Scheidt, 1998; Raila et al., 2002). As further support of this idea, American goldfinches fed concentrated supplements of β-carotene failed to incorporate this pigment into feathers to grow orange plumage (McGraw et al., 2001).

However, given the fact that a dietary xanthophyll such as lutein was present in feathers, the absence of zeaxanthin was unexpected. As indicated above, zeaxanthin is nearly always found in conjunction with lutein in both photosynthetic organisms and animals (Goodwin, 1980, 1984). One possible explanation for the absence of zeaxanthin in these feathers is that it was destroyed during the thermochemical pigment-extraction process. However, when we subjected pure, crystalline zeaxanthin (donated by R. Stradi, University of Milan, Italy) to the acidified-pyridine treatment, it was recovered fully (with no modification to lutein or its isomers). Moreover, using this same extraction technique, we have previously isolated zeaxanthin from the feathers of other songbirds (e.g. the red-winged blackbird, *Agelaius phoeniceus*; K.J.M., unpublished data). Thus, we are confident that lutein is the only carotenoid present in the yellow feathers under study. Consequently, this result indicates post-absorptive selective accumulation of lutein (over zeaxanthin) in yellow feathers. We are unaware of a parallel system of specific hydroxycarotenoid discrimination in vertebrates.

We further investigated this process of lutein sequestration and zeaxanthin exclusion in yellow-feathered passerines by sampling carotenoids at the site of feather growth. Since blood delivers nutrients to maturing feathers, and yet blood did not solely contain lutein, we suspected that the developing feather was the site of pigment discrimination. By plucking immature, still-growing yellow feathers in two of the study species, we obtained yellow-colored lipid fractions (containing carotenoids) that were not yet keratinized into feathers. We found that this material contained both lutein and zeaxanthin, in a ratio that was nearly identical to that found in the blood of these molting birds. These data suggest that the maturing follicle exerts local control over pigment incorporation into bird feathers (*sensu* Giersberg and Stadie, 1933; Brockmann and Völker, 1934), in this case by preferentially excluding zeaxanthin.

This regulatory feat is probably accomplished by a binding protein located in follicular cells. Numerous carotenoid-binding proteins (or carotenoproteins) have been characterized in plants (Lakshman and Okoh, 1993; Reddy et al., 1993) and invertebrates (Zagalsky, 1995), including the well-known crustacyanin complex in lobster (Keen et al., 1991). Of relevance to selective hydroxycarotenoid retention in this study is the lutein-binding protein in the gut (Jouni and Wells, 1996) and the silk gland (Tabunoki et al., 2002) of the silkworm (*Bombyx mori*) and the recently characterized xanthophyll-binding protein that facilitates accumulation and retention of xanthophylls in the human macula/retina (Yemelyanov et al., 2001). However, in these instances, it is not apparent that any protein is specific to lutein and not to zeaxanthin. Despite being termed a 'lutein-binding protein', no information is available on the binding capacity of zeaxanthin to the silkworm carotenoprotein (K. Tsuchida, pers. comm.). Moreover, the macular protein in human retina selectively harbors both lutein and zeaxanthin (but exhibits no binding activity toward canthaxanthin, a ketocarotenoid, and β-carotene, an unsubstituted carotenoid). Cellular, genetic and immunological work is needed to determine if a unique binding protein with exquisite specificity for lutein exists in bird feathers.

Why might these birds selectively incorporate lutein into and exclude zeaxanthin from feathers? From a mechanistic standpoint, there may be molecular interactions between carotenoids, as they compete for limited access to micelles during absorption or to protein binding sites during tissue deposition (van den Berg, 1999); in this case, lutein may outcompete zeaxanthin for binding sites in feather follicles. At the functional level, it is especially perplexing why a more conjugated (and thus more colorful) molecule like zeaxanthin is not shunted to brightly colored feathers that probably are used as sexual or social signals to attract mates or repel competitors. One intriguing possibility is that with its more conjugated state comes superior antioxidant activity over lutein (e.g. Mortensen and Skibsted, 1997), such that zeaxanthin may be saved in living tissue for intracellular free-radical-scavenging rather than made inaccessible by allocating it to non-living feathers. Another idea is that extrinsic selection pressures such as predation and light environment (e.g. Andersson, 2000) favor yellow lutein-based plumage coloration over the more orange hue that would be generated by the incorporation of zeaxanthin into feathers.

In the end, the fact that species from three different songbird families (Lovette and Bermingham, 2002) follow this system of pigmentation suggests that it is a rather conserved trait among New World passerines (parvorder Passerida, superfamily Passeroidea). We encourage more biochem-

ical work in other yellow-colored birds across the world to determine how widespread this form of carotenoid discrimination truly is.

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