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CAROTENOID PIGMENTS IN A MUTANT CARDINAL: IMPLICATIONS FOR THE GENETIC AND ENZYMATIC CONTROL MECHANISMS OF CAROTENOID METABOLISM IN BIRDS

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Abstract. Birds that use carotenoids to color their feathers must ultimately obtain these pigments from the diet, but they are also capable of metabolically transforming dietary carotenoids into alternate forms that they use as plumage colorants. The genetic and enzymatic control mechanisms underlying carotenoid metabolism are poorly understood. We investigated carotenoid pigments present in the feathers of an aberrantly colored yellow Northern Cardinal (*Cardinalis cardinalis*) to determine how metabolic pathways may have been altered. Normal red cardinals display four primary keto-carotenoids in plumage that are endogenously derived from a series of common food carotenoids. We found that the yellow feathers of this mutant lacked all four of the typical red pigments, and instead contained a yellow dietary carotenoid (lutein) and three yellow metabolic derivatives (3′-dehydrolutein and canary-xanthophylls A and B). Because yellow metabolites appear to be manufactured via a different metabolic process (dehydrogenation) than the usual red forms (oxidation at the C-4 or -4′ positions), it seems that this genetic mutation did not broadly disrupt all metabolic activity, but specifically impaired only one class of metabolic reactions and its associated enzymes.

Key words: Cardinalis cardinalis, *carotenoids, metabolism, mutation, Northern Cardinal.*

Pigmentos Carotenoides en un Cardenal Mutante: Implicaciones para los Mecanismos Genéticos y Enzimáticos de Control del Metabolismo de Carotenoides en las Aves

Resumen. Las aves que utilizan pigmentos carotenoides para dar color a sus plumas deben obtenerlos de la dieta, pero también son capaces de transformar metabólicamente los carotenoides dietarios en formas alternativas que utilizan para colorear las plumas. Los mecanismos genéticos y enzimáticos de control involucrados en el metabolismo de los carotenoides no han sido bien establecidos. En este estudio investigamos los pigmentos carotenoides presentes en las plumas de un cardenal *Cardinalis cardinalis* con coloración amarilla aberrante para determinar cómo se podrían haber alterado las rutas metabólicas. Los cardenales normales de color rojo despliegan cuatro keto-carotenoides primarios en el plumaje, los cuales son derivados endógenamente a partir de una serie de carotenoides comunes en la dieta. Encontramos que las plumas amarillas del mutante carecían de los cuatro pigmentos rojos típicos, y contenían en cambio un carotenoide dietario amarillo (luteína) y tres derivados metabólicos amarillos (3′-dehidro luteína y xantofilas de canario A y B). Debido a que los metabolitos amarillos parecen ser fabricados mediante un proceso metabólico diferente al de las formas rojas usuales (deshidrogenación y oxidación en las posiciones C-4 o -4′, respectivamente), parece que esta mutación genética no alteró ampliamente toda la actividad metabólica. En cambio, parece haber inhabilitado sólo una clase de reacciones metabólicas y sus enzimas asociadas.

For nearly a century, biologists have known that carotenoid pigments cannot be synthesized *de novo* by vertebrates (Palmer 1922). Instead, carotenoids must be obtained in the diet to serve various physiological functions or to be used in integumentary color displays (Olson and Owens 1998, Hill 1999, Møller et al. 2000). Animals can, however, metabolically alter ingested pigments (Davies et al. 1970, Brush 1990, Schiedt 1990). Birds, for example, consume a variety of dietary carotenoids, including the hydroxycarotenoids lutein and zeaxanthin, but often deposit into feathers and bare parts more oxidized forms, such as the canary-xanthophylls that are yellow in color or red keto-carotenoids like astaxanthin (Stradi 1998).

Despite recent interest in the proximate and ultimate bases for variation in carotenoid pigmentation in birds (Hill 2002), the particular enzymatic and genetic mechanisms controlling avian carotenoid metabolism remain essentially undescribed. Only in domestic chickens (*Gallus domesticus*) have dietary carotenoids been radiolabeled *in vivo* to determine the metabolic fate of ingested precursor pigments in birds (Schiedt 1990, 1998). Moreover, only in studies of β-carotene as a vitamin-A precursor have enzymes been identified that catalyze carotenoid metabolism in animals (Wyss

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FIGURE 1. Reference photograph of the mutant yellow cardinal, placed next to a museum specimen of a wild-type red cardinal.

et al. 2000), and this central-cleavage process is much different from the end-ring oxidation reactions that are more common in birds (Stradi 1998, Stradi et al. 2001). Ultimately, to better understand how and why birds complete these pigment transformations, more consideration should be given to the biochemical and genetic systems that regulate the enzyme(s) that metabolize dietary carotenoids.

Studies of mutant individuals that fail to display the species-typical plumage-color pattern (e.g., Völker 1964, Hudon 1997) may provide valuable insights into the genetic and enzymatic systems of carotenoid metabolism in colorful birds. In this study, we compared the carotenoid-pigment profile in the plumage of an aberrantly colored male Northern Cardinal (*Cardinalis cardinalis*) to that of wild-type individuals to understand the degree to which changes in metabolic pathways generated this plumage-color variant. Wild-type male Northern Cardinals are bright red, and their red feather carotenoids are synthesized endogenously from the yellow and orange precursor pigments they obtain from the diet (McGraw et al. 2001). However, in July 1989, an adult male cardinal with uniformly yellow plumage was collected in Baton Rouge, Louisiana, by the Museum of Natural Science at Louisiana State University (museum specimen # LSUMZ 139415; Fig. 1). To our knowledge, this is the lone reported yellow cardinal in North America. Even when fed carotenoid-deprived diets in captivity during molt, male cardinals still grow a pale red plumage, manufacturing the typical suite of red keto-carotenoids from the low concentration of available dietary carotenoids (McGraw et al. 2001). Thus, it seems that a deficiency of dietary carotenoids should not have induced this individual to grow yellow feathers, but instead that this bird may have lost its ability to metabolize carotenoids from the diet through the disruption of genetically determined metabolic pathways. We obtained a sample of yellow contour feathers from this specimen and used high-performance liquid chromatography (HPLC) to determine its plumage-carotenoid composition so that it could be compared to the pigment profile of wild male cardinals (McGraw et al. 2001).

METHODS

In April 2001, we obtained 6 pigmented contour feathers from this yellow male Northern Cardinal from the LSU Museum of Natural Science. These feathers were divided into two groups of three, and the carotenoids were extracted from each using two different methods: a thermochemical procedure (Hudon and Brush 1992), and a mechanical procedure (Stradi, Celentano, and Nava 1995). Prior to each procedure, we separately washed the feathers in 5 mL ethanol and 5 mL hexane for 30 min, blotted them dry, and trimmed off the carotenoid-pigmented barbules for analysis. For the thermochemical method, we added 1 mL acidified pyridine to the barbules in a 9-mL glass tube, filled the headspace with argon, and held the solution at 95°C for 3 hr. The tube was then cooled to room temperature and we extracted the lipids by adding 1 mL distilled water and 5 mL hexane:*tert*-butyl methyl ether (1:1) and shaking vigorously for 2 min. For the mechanical method, we ground pigmented barbules for 15 min at 30 Hz using a Retsch® MM200 mixer mill (Retsch Inc., Irvine, California), equipped with a zirconia grinding jar and balls (and Teflon® O-ring) and in the presence of 3 mL methanol. At this point in both procedures, we centrifuged the resulting mixtures for 5 min at 3000 rpm, transferred the supernatant to a clean tube, and evaporated the solvent to dryness under a stream of nitrogen. The pigment residue was resuspended in 200 µL of HPLC mobile phase (methanol:acetonitrile:chloroform, 46:46:8) prior to analysis.

We injected 50 µL of each sample into a Waters™ 717plus Autosampler HPLC (Millipore Corp., Bedford, Massachusetts) fitted with a Develosil RPAqueous RP-30 column (250 × 4.6 mm; Nomura Chemical Co. Ltd., Aichi, Japan) and an Eppendorf TC-50 column heater (Hamburg, Germany) set at 27°C. We used an isocratic system (Hewlett-Packard 1050 Series Isocratic Pump) at a constant flow rate of 0.6 mL min^{-1} for 60 min to allow sufficient time for both xanthophylls and carotenes to elute if they were present. Data were collected from 250–600 nm using a Waters™ 996 photodiode array detector (Waters Corporation, Milford, Massachusetts). We identified pigments by comparing their respective retention times and absorbance maxima to those of authentic reference carotenoids run as external standards. The relative abundance of these various carotenoids was determined by comparing peak areas at 450 nm using Millenium™ software (Waters Corporation 1991). We used similar pigment analyses for wild-type male cardinals; see McGraw et al. (2001) for details.

RESULTS

In earlier work, we demonstrated that normal male Northern Cardinals display four primary red keto-carotenoids in their colorful plumage: α-doradexanthin, astaxanthin, adonirubin, and canthaxanthin (McGraw et al. 2001). These occur in feathers at an approximate ratio of 19:35:30:16, respectively, and at concentrations ranging from 0.5–2 mg pigment per gram of pigmented feather barbule (KJM, unpubl. data).

We found that none of these red carotenoids were present in the yellow feathers of the mutant cardinal. Instead, we detected two primary HPLC peaks, comprising 90% of all signals, at 13.1 and 13.4 min, with each having two absorption maxima: at 445 nm and 474 nm. By comparison to authentic reference carotenoids, we identified these two pigments as lutein (β,ε-carotene-3,3′-diol) and 3′-dehydrolutein (also known as 3′-ketolutein, or 3-hydroxy-β,ε-caroten-3′-one; Fig. 2). Lutein was the more concentrated of the two plumage carotenoids, comprising 54% of all feather pigments, whereas 3′-dehydrolutein made up 37% of the total. There were also two minor peaks, together amounting to ca. 10% of plumage carotenoids, with elution times of 9.4 and 9.7 min. These two eluents matched our standards for canary-xanthophyll B (3′-hydroxy-ε,ε-caroten-3-one) and A (ε,ε-caroten-3,3′-dione), respectively (Fig. 2).

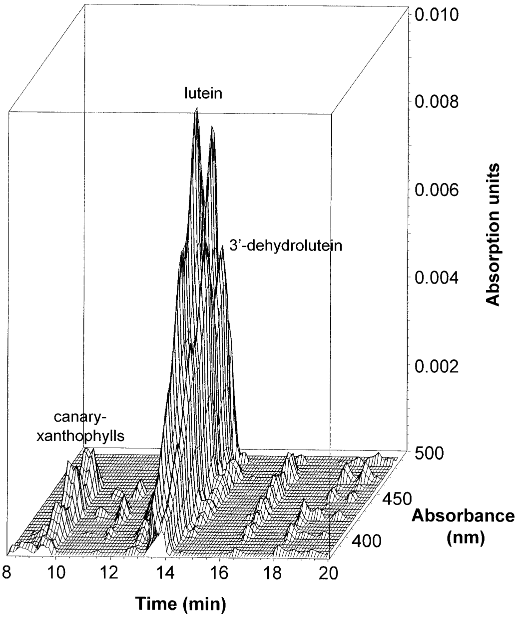

FIGURE 2. Three-dimensional HPLC chromatogram of the carotenoid pigments present in the mutant yellow plumage of a male cardinal. Lutein and 3′-dehydrolutein were the major plumage carotenoids, comprising 90% of total eluents, whereas canary-xanthophylls A and B were minor constituents. Total carotenoid concentration in mutant feathers was 0.73 mg g^{-1}, which falls within the range of variation observed in wild red cardinals and other carotenoid-pigmented songbirds (KJM, unpubl. data). See McGraw et al. (2001) for comparable chromatograms from wild-type Northern Cardinals.

Samples from both the thermochemical and mechanical extraction procedures yielded identical plumage-carotenoid profiles. Because carotenoids may isomerize or degrade over time with exposure to light and oxygen, we tested the stability of plumage pigments in museum skins by obtaining red feathers from wild-type specimens that were older (collected in 1964 and 1970) than the mutant cardinal studied here. We found the expected set of red carotenoids in these feathers, indicating that the pigment profile in this yellow cardinal is also unlikely to have changed since the time of collection.

DISCUSSION

Northern Cardinals manufacture the red carotenoid pigments that they deposit in feathers from a suite of four yellow or orange carotenoids present in the diet (lutein, zeaxanthin, β-cryptoxanthin, and β-carotene; McGraw et al. 2001). The presumed metabolic processes that form these red plumage colorants are a series of oxidation reactions at the C-4 or -4′ positions (hereafter, 4-oxidation), where one or two keto groups are introduced to the β-ionone rings of the different dietary carotenoid molecules (Fig. 7 in McGraw et al. 2001). Such oxidative transformations appear to be common in songbirds with red plumage (Stradi 1998).

Here, we studied the carotenoid composition of feathers from an aberrant wild cardinal, displaying yellow plumage pigmentation, to understand the metabolic changes accompanying this unusual coloration. Specifically, we were interested in determining whether this bird had lost the ability to transform all dietary carotenoids or if there were particular metabolic pathways that were altered. We found that this cardinal did not incorporate into its plumage any of the red keto-carotenoids that typically give males their red color. Instead, the common dietary carotenoid lutein was the main pigment in its yellow feathers, as is found in other yellow-colored birds that deposit dietary carotenoids directly into plumage (e.g., tits, Old World orioles, wagtails; Stradi 1998).

If we assume that this bird had access to a diet that was typical of cardinals, containing the set of dietary carotenoids listed above, it seems that this yellow cardinal could not manufacture any of the four keto-carotenoids normally found in the red feathers of cardinals. This does not appear to be an unreasonable assumption, as most fruits and seeds contain a mix of these carotenoids (Goodwin 1980) and because a representative sample of seeds and insects was found in the crop of this animal at the time of collection (V. Remsen, pers. comm.). The fact that lutein was the predominant plumage carotenoid is most likely a product of its abundance relative to other xanthophylls and carotenes in the vegetable diet (Goodwin 1980, Mangels et al. 1993), combined with the fact that birds preferentially accumulate the polar hydroxy- and keto-carotenoids over nonpolar molecules like β-carotene (Schiedt 1998). Ultimately, because this cardinal did not synthesize any of the typical red feather carotenoids, it seems that it was generally unable to perform these oxidation reactions at the C-4 or -4′ positions. Stradi et al. (1996) has proposed that a single generalist enzyme, 4-oxygenase, is responsible for the formation of these 4-keto-carotenoids in birds, regardless

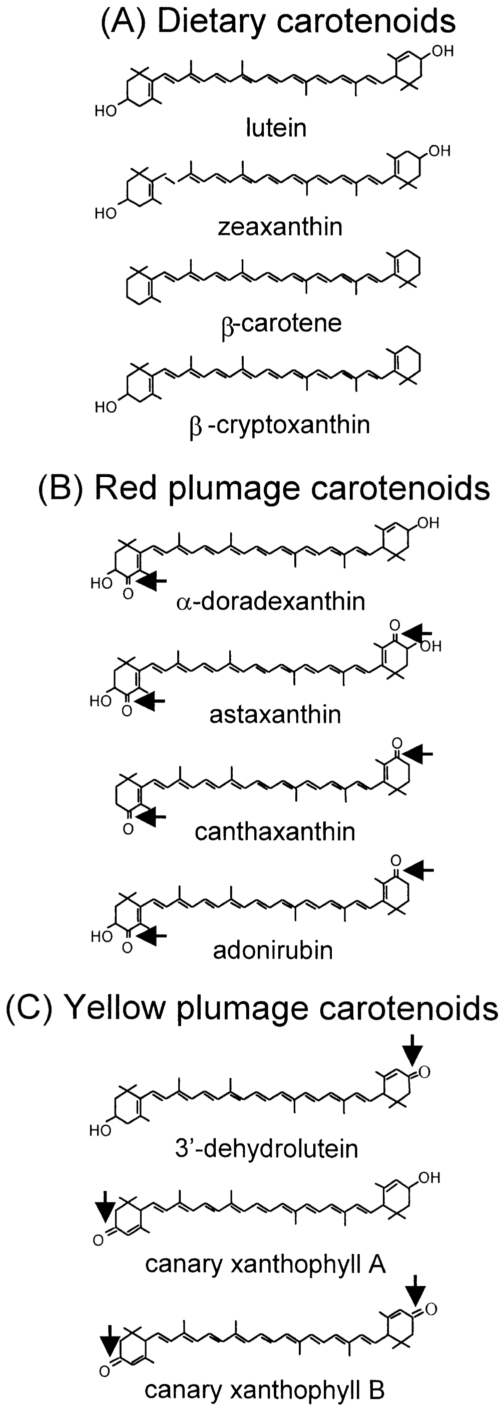

FIGURE 3. Structural formulas for the carotenoid pigments found in the diet and feathers of Northern Cardinals: (A) yellow and orange carotenoids ingested in the diet, (B) red pigments found in the plumage of wild-type cardinals (McGraw et al. 2001), and (C) yellow pigments present in the feathers of the aberrant yellow cardinal studied here. Hydroxylated or unsubstituted carotenoids occur in the basic diet of these birds, and normal cardinals metabolize these dietary compounds via the addition of one or two keto groups (denoted by arrows in B) at the C-4 position of the β-ionone ring, into four red 4-keto-carotenoids. However, the yellow mutant failed to incorporate any of these red colorants into feathers, and instead deposited dietary pigments directly (e.g., lutein) or manufactured yellow carotenoids via a different oxidation process than the predominant pathway in red cardinals. This process of dehydrogenation involves the conversion of hydroxyl groups to carbonyls at the C-3 position on the end-rings of the carotenoid molecule (denoted by arrows in C).

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of the degree of β-ionone-ring substitution in the precursor (see also mixed-function-oxidase in Hudon 1994). Our findings suggest that a genetic, loss-of-function mutation in this cardinal impaired oxygenase activity and knocked out its ability to complete such enzyme-catalyzed reactions.

Interestingly, three other carotenoids (3′-dehydrolutein and canary-xanthophylls A and B) were also present in the yellow plumage of this mutant cardinal. 3′-dehydrolutein is an uncommon yellow carotenoid in bird feathers (e.g., thus far found only in the Red-billed Leiothrix [*Leiothrix lutea*], Stradi et al. 1996; Pine Grosbeak [*Pinicola enucleator*], Stradi 1998; House Finch [*Carpodacus mexicanus*], Inouye et al. 2001), whereas the canary-xanthophylls are some of the more common yellow carotenoids in bird plumage, found in the tail feathers of waxwings (*Bombycilla* spp.; Hudon and Brush 1989, Stradi 1998) and the body plumage of *Carduelis* finches, for example (Stradi, Celentano, et al. 1995, McGraw et al. 2001, 2002). As dehydrolutein and the canary-xanthophylls have never been reported in avian diets, we presume they are of metabolic origin, putatively derived from both lutein and zeaxanthin (Stradi, Celentano, et al. 1995). In fact, the chemical formation of 3′-dehydrolutein from zeaxanthin has been confirmed with pigment radiolabeling experiments *in vivo* with chickens (Schiedt 1998), and this pathway could explain why no zeaxanthin was found in the feathers of this mutant cardinal.

Thus, although dietary lutein was the major plumage pigment in this yellow cardinal, this bird was still able to perform certain metabolic conversions of ingested carotenoids. In contrast to the aforementioned 4-oxidation reactions that form red keto-carotenoids in normal cardinal plumage, the hypothesized pathways that produce 3′-dehydrolutein and the canary-xanthophylls in this yellow cardinal all involve the process of dehydrogenation (i.e., the oxidation of hydroxyl groups to carbonyls; Fig. 3; Stradi 1998). Along with 4-oxidation reactions, dehydrogenation is also a very common metabolic transformation among colorful songbirds, particularly those with yellow plumage (Stradi 1998). Nevertheless, the prevalence of these yellow metabolites in mutant-cardinal feathers, but not in the plumage of wild-type red cardinals, suggests a shift in metabolic activity due to this genetic mutation, from 4-oxidation reactions to dehydrogenation of hydroxylated precursors.

This difference in metabolic activity has also been described in a lineage of tanagers (*Piranga*), in which the Western Tanager (*P. ludoviciana*) forms yellow canary-xanthophylls rather than the red 4-keto-carotenoids that predominate in red species (e.g., Scarlet Tanager, *P. olivacea*; Hudon 1991). Hudon (1991) has also reported minor concentrations of at least one of the canary-xanthophylls in the red plumage of wild Northern Cardinals, suggesting that dehydrogenation pathways exist in normal cardinals but are not the preferred or predominant ones. We suspect that, by impairing 4-oxidation processes, this genetic mutation resulted in the expression of alternate dehydrogenation reactions and their associated enzymes (dehydrogenases), which formed plumage colorants that do not appear in high concentrations in normal cardinal feathers because of higher enzyme titers of or substrate affinity for the oxygenase(s) that produce red keto-carotenoids. Due to the difficulty in transforming these yellow dehydrogenated products into the red 4-keto-carotenoids typically found in cardinal feathers (e.g., by replacing keto with hydroxy groups), it is likely that these two metabolic pathways exist independently in the cardinal, rather than as sequential steps that lead to the formation of final red plumage pigments.

Interestingly, recent characterization of plumage carotenoids in a mutant form (yellow mask) of the Gouldian Finch (*Chloebia gouldiae*) shows that both lutein and 3′-dehydrolutein are present in yellow feathers that are normally colored red and contain 4-keto-carotenoids (Massa and Stradi 1999). Thus, the genetically altered pathway that we have elucidated here may be a conserved feature among certain carotenoid-colored species. Future studies aimed at identifying carotenoid-metabolizing enzymes and the genes that encode them, as has been done in plants, algae, fungi, and bacteria (e.g., Verdoes et al. 1999, Velayos et al. 2000), will undoubtedly improve our understanding of the intrinsic processes that govern carotenoid pigmentation in colorful birds.

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