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Carotenoid pigments and the selectivity of psittacofulvin-based coloration systems in parrots

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Abstract

Carotenoid pigments are commonly used as colorants of feathers and bare parts by birds. However, parrots (Aves: Psittaciformes) use a novel class of plumage pigments (called *psittacofulvins*) that, like carotenoids, are lipid-soluble and red, orange, or yellow in color. To begin to understand how and why parrots use these pigments and not carotenoids in their feathers, we must first describe the distribution of these two types of pigments in the diet, tissues, and fluids of these birds. Here, we studied the carotenoid content of blood in five species of parrots with red in their plumage to see if they show the physiological ability to accumulate carotenoids in the body. Although Scarlet (*Ara macao*) and Greenwing Macaws (*Ara chloroptera*) and Eclectus (*Eclectus roratus*), African Gray (*Psittacus erithacus*) and Blue-fronted Amazon (*Amazona aestiva*) Parrots all use psittacofulvins to color their feathers red, we found that they also circulated high concentrations of both dietary (lutein, zeaxanthin, β-cryptoxanthin) and metabolically derived (anhydrolutein, dehydrolutein) carotenoids through blood at the time of feather growth, at levels comparable to those found in many other carotenoid-colored birds. These results suggest that parrots have the potential to use carotenoids for plumage pigmentation, but preferentially avoid depositing them in feathers, which is likely under the control of the maturing feather follicle. As there is no evidence of psittacofulvins in parrot blood at the tune of feather growth, we presume that these pigments are locally synthesized by growing feathers within the follicular tissue.

Keywords: Carotenoid metabolism; Carotenoids; HPLC; Lutein; Macaw; Plasma carotenoids; Plumage coloration; Psittaciformes; Psittacofulvins; Zeaxanthin

1. Introduction

Birds use several classes of pigments to become colorful (Fox and Vevers, 1960). Melanin pigments most commonly appear in bird feathers and bare parts and confer black, brown, gray, and chestnut hues (Prota, 1992). Carotenoid pigments are a second, well-studied group of colorful biochemicals in birds and bestow brilliant red, orange, and yellow colors on the integument (Stradi, 1998). These two types of pigment-based coloration are found in nearly every order of extant birds (K.J. McGraw, personal observation).

There are some exceptions to this rule, however. Parrots (Class Aves, Order Psittaciformes), for example, display a spectrum of red-to-yellow hues in their feathers, but do not use carotenoids to generate these colors (Völker, 1936, 1937, 1942; Hudon and Brush, 1992). Although we have known this fact for over a century, when the name *psittacofulvins* was given to this unique class of biochromes in parrots (Krukenberg, 1882), the actual biochemical nature of these compounds proved elusive until recently (Veronelli et al., 1995; Stradi et al., 2001). Moreover, we still lack an understanding, from both a mechanistic and a functional perspective, as to why parrots use these novel pigments as feather colorants.

To begin to understand the proximate and ultimate causes of psittacofulvin-based pigmentation in parrots, we must describe the availability and distribution of these pigments in these birds. Carotenoids and psittacofulvins not only share similar light-reflectance characteristics (based on their extended chains of C=C bonds; Veronelli et al., 1995), but also have similar solubility (e.g., lipophilic) properties (Hudon and Brush, 1992) and should be equally likely to be incorporated into feathers, since lipids are thought to

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passively diffuse from circulation into maturing follicles (Lucas and Stettenheim, 1972). Ultimately, to use psittacofulvins and not carotenoids as plumage colorants, parrots must either not accumulate carotenoids in the body or they must show a specific affinity for psittacofulvins (or exclusion of carotenoids) at some step in the pigmentation process. Animals acquire carotenoids from dietary sources (e.g., plant matter, herbivorous prey) and deliver them through the bloodstream to peripheral tissues for pigmentation (reviewed in McGraw and Hill, 2001). At present, the physiological or anatomical origins of psittacofulvins are unknown (Stradi et al., 2001), although it is believed that dietary precursors do not play a significant role in the acquisition of colorful parrot plumage (Völker, 1936).

Thus, we studied the lipochrome composition of blood in five species of red-colored parrots, at the time in which birds were growing their colorful psittacofulvin-based plumage, to determine (a) if they have the physiological ability to accumulate carotenoids in the body and (b) where psittacofulvins may originate in the body.

2. Methods

2.1. Study species

We studied single mated adult pairs of Eclectus Parrots (*Eclectus roratus*; each 7 years old), African Gray Parrots (*Psittacus erithacus*; 7-year-old female and 5-year-old male) and Scarlet Macaws (*Ara macao*; 14-year-old female and 7-year-old male), as well as a 14-year-old male Blue-fronted Amazon Parrot (*Amazona aestiva*) and a 7-year-old female Greenwing Macaw (*Ara chloroptera*). All five species display some degree of red coloration in their plumage (Forshaw, 1977). Female Eclectus Parrots and Scarlet and Greenwing Macaws color most of their body with red feathers. Male Eclectus Parrots display red-pigmented flanks and underwing coverts. African Grays grow red undertail coverts and retrices, whereas Blue-fronted Amazons restrict red pigment patches to the shoulders, wings, and tail.

2.2. Plasma analyses

Blood was collected from each of these pet birds by their respective veterinarians. Plasma was centrifuged off and stored at −20 °C in 1.5 ml microcentrifuge tubes for 1–7 days before being shipped on dry ice to KJM. Tubes were then stored at −80 °C.

The two classes of lipochromes under study—psittacofulvins and carotenoids—have similar solubilities (but different light-absorbance properties; Hudon and Brush, 1992; Stradi et al., 2001); therefore, we were able to use the same, previously published protocol (McGraw et al., 2002, 2003a) to extract and analyze both types of pigments in plasma. To a fresh tube containing 10 μl thawed plasma, we added 75 μl ethanol and 75 μl *tert*-butyl methyl ether. The tube was vortexed for 5 s and centrifuged for 4 min at 16,000 × *g*. We transferred the supernatant to a fresh HPLC vial and evaporated the solvent to dryness under a stream of nitrogen. We resuspended the pigment residue in 200 μl HPLC mobile phase (46:46:8, methanol/acetonitrile/chloroform, v/v/v) and injected 50 μl into a Waters™ 717plus autosampler HPLC (Millipore, Milford, MA, USA) fitted with a Develosil RPAqueous RP-30 column (250 × 4.6 mm i.d.; Nomura Chemical, Japan) and an Eppendorf TC-50 column heater (set at 31 °C). An isocratic system (HP 1050 Series Isocratic Pump) was run for 45 min at a constant flow rate of 1.2 ml/min to detect both psittacofulvins and carotenoids (xanthophylls and carotenes) if present. Pigments were identified by comparison to external standards (Scarlet Macaw feather pigments for psittacofulvins, see below for carotenoids) and quantified using an internal standard of known concentration (1 μg/ml canthaxanthin) that we previously determined to be absent from the plasma of these birds.

We measured plasma-carotenoid concentrations on two occasions (13 May and 12 September 2003) and found them to be highly repeatable (Lessells and Boag, 1987) within birds ($R_i = 0.98$, $F_{7,8} = 79.3$, $p < 0.0001$). Thus, we report means of the two values for all individuals.

3. Results

In a previous study, we followed the analytical methods of Stradi et al. (2001) to investigate the pigmentary basis of red plumage coloration in the five parrot species under study. Akin to the pigment-based coloration of Scarlet Macaws, we showed using HPLC that Greenwing Macaws and Eclectus, African Gray, and Blue-fronted Amazon Parrots all use the same suite of psittacofulvins (not carotenoids) to color their feathers red (McGraw and Nogare, unpublished data).

In this study, we investigated the pigment composition of blood in these parrots to determine whether carotenoids and/or psittacofulvins are circulated through blood during the time of feather growth. Using HPLC, we detected a series of five main carotenoids in the blood of all species studied (Fig. 1). By comparison to the retention times (t_R) and absorbance maxima (λ_{max}) of authentic reference standards that were donated by Dr. Riccardo Stradi (University of Milan, Italy), Dr. Fred Khachik (University of Maryland, USA), and Roche Vitamins (Parsippany, NJ, USA), we identified these blood carotenoids as (all in *trans* form): lutein ($t_R = 7.2$ min, $\lambda_{max} = 448$ nm), zeaxanthin ($t_R = 7.5$ min, $\lambda_{max} = 453$ nm), 3′-dehydrolutein ($t_R = 7.9$ min, $\lambda_{max} = 448$ nm), 2′,3′-anhydrolutein ($t_R = 15.8$ min, $\lambda_{max} = 448$ nm), and β-cryptoxanthin ($t_R = 21.1$ min, $\lambda_{max} = 453$ nm) (Fig. 1). *cis* Lutein and zeaxanthin isomers also were present at $t_R = 9.4$ and 10.3 min, respectively. Concentrations of these pigments varied among and within

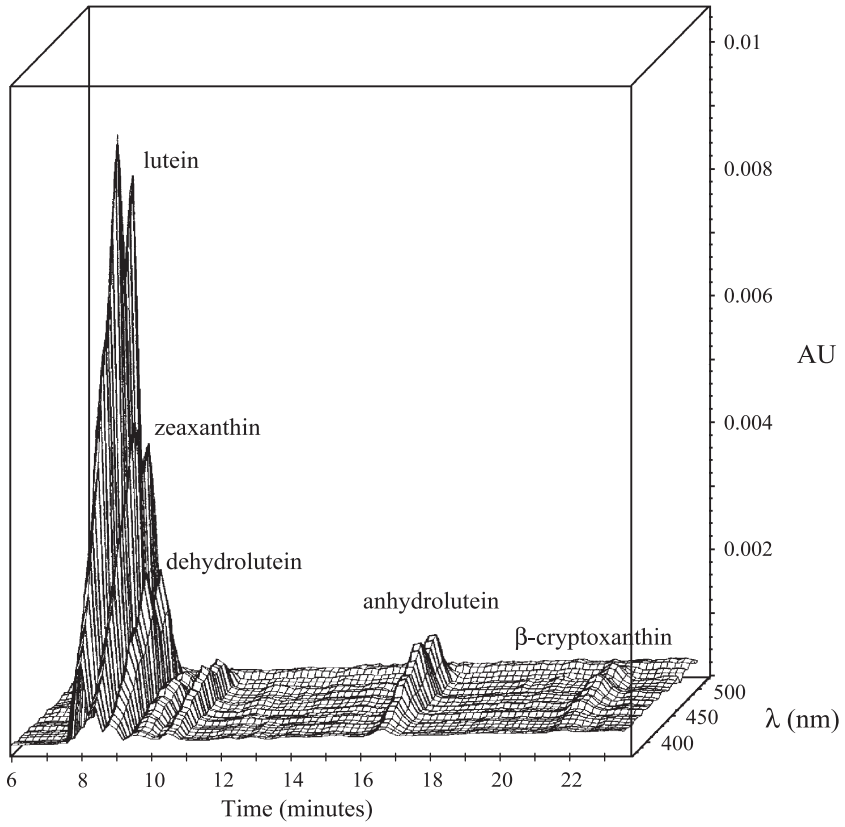

Fig. 1. Representative three-dimensional HPLC chromatogram depicting the carotenoids found in the blood plasma of parrots: female African Gray Parrot (*Psittacus erithacus*) shown.

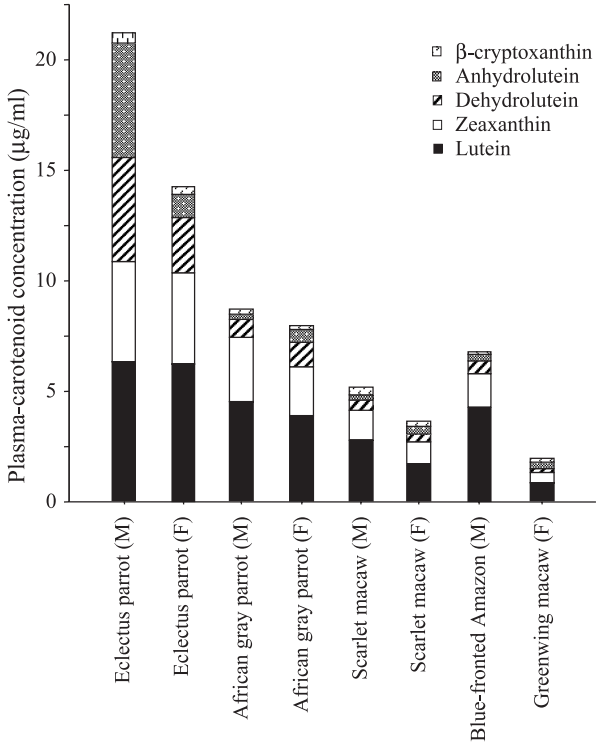

Fig. 2. Histogram showing the concentration of different plasma carotenoids from the eight parrots under study.

species (Fig. 2), but lutein and zeaxanthin were dominant, comprising a combined 74.0 ± 10.9% of total (mean ± S.D.). Overall carotenoid concentrations ranged from 2 to 22 μg/ml across the individuals sampled (Fig. 2). These same five carotenoids were also found in the yolks of two eggs laid by the pair of Eclectus Parrots studied here (data not shown).

However, our analyses of plasma lipochromes yielded no evidence of psittacofulvins in parrot blood. The detection limit of our analytical system is 1 ng pigment per 50 μl injection.

4. Discussion

Several biochemical and physiological aspects of parrot pigmentation have yet to be described. We took a first step at understanding the specificity of psittacofulvin-based coloration in parrots by documenting the types of lipochromes present in blood. We detected a suite of five carotenoid pigments circulating through the body of these captive parrots. These birds naturally acquire carotenoids from their vegetable diets (e.g., seeds, fruits), and the captive parrots under study here were fed diverse diets consisting of nuts, seeds, fruits, vegetables, along with a formulated parrot mix (M.C. Nogare, personal observation). Thus, even though they fail to use them in plumage, parrots

have the capacity to accumulate carotenoids in the body, at levels comparable to many previously studied birds. For example, carotenoid concentrations in blood spanned two orders of magnitude (from 0.75 to 74 μg/ml) in a comparative study of 26 avian species representing seven orders and fourteen families (no psittaciformes were included; Tella et al., 1998). Carotenoid-pigmented species like the American Kestrel (*Falco sparverius*) and the White Stork (*Ciconia ciconia*) respectively circulate 5–25 μg/ml (Tella et al., 1998) and 8 μg/ml plasma carotenoids, on average (Negro and Garrido-Fernandez, 2000). While it is difficult to compare the true carotenoid demands for integumentary pigmentation across species (without knowing rates of pigment turnover and uptake by feathers), it is still clear that these parrots have the potential to incorporate at least some carotenoids into feathers.

In contrast, we did not find any psittacofulvins in blood, indicating that these pigments are not acquired from the diet directly by these parrots. This idea is supported by the fact that parrots do not change in color when housed in captivity on a wide variety of diets (Völker, 1936; Nemesio, 2001). These results also suggest that red psittacofulvins are not manufactured at central sites in the body like the liver or small intestine for delivery to peripheral tissues through the bloodstream. Instead, we are left with the notion that parrots locally synthesize these compounds at growing feathers, within the maturing feather follicle. This is a common phenomenon for plumage pigments, as melanins are synthesized by melanocytes within feather tracts (Ralph, 1969), and as it has been hypothesized that metabolically derived 4-oxo-carotenoids are also synthesized at the feather follicle (Stradi, 1998, p. 56).

Taken together, these observations on lipochrome distribution in tissues and fluids generate the following alternative hypotheses regarding the specificity of parrot pigmentation systems: (a) carotenoids are selectively excluded from parrot feather follicles at the extracellular level, or (b) both types of lipid-soluble pigments are present in follicles, but the binding-site specificity of intracellular proteins is such that psittacofulvins are preferentially keratinized into feathers over carotenoids. The general model for lipid delivery to maturing feathers supports hypothesis (b), as lipids are believed to diffuse passively into feathers via the formation of lipoidal droplets (Lucas and Stettenheim, 1972; Menon and Menon, 2000). A prior study of selective carotenoid incorporation into songbird feathers also pointed to an intracellular mode of preferential pigment uptake (McGraw et al., 2003b). Future studies of the pigmentary composition of these lipoidal droplets in growing parrot feathers could be useful for distinguishing between these two hypotheses.

Blood-carotenoid concentrations often differ between the sexes in birds, with males typically showing higher levels than females (reviewed in McGraw et al., 2003a). This has been shown in avian species with sexually dichromatic carotenoid-based integumentary coloration (e.g., American Kestrels) as well as in species that fail to display any carotenoid coloration in feathers or bare parts (e.g., Loggerhead Shrikes [*Lanius ludovicianus*]; Bortolotti et al., 1996). Although the sample sizes in this study are too small to conduct statistical tests, it is curious that male parrots had higher plasma-carotenoid levels than females in all three species in which we measured blood carotenoids for both sexes. As these parrots do not use carotenoids as body colorants, these results point to alternate, perhaps physiological functions of carotenoids (Lozano, 1994). Carotenoids serve as potent antioxidants and immunostimulants in birds and other animals (reviewed in McGraw and Ardia, 2004), and it is possible that males accumulate higher carotenoid levels than females to combat testosterone-induced immunosuppression that is so common in male animals (McGraw and Ardia, unpublished data).

The presence of metabolically derived carotenoid pigments in parrot plasma also warrants further consideration. Based on prior studies of diet and plasma carotenoids in granivorous and frugivorous birds (e.g., Slifka et al., 1999; McGraw et al., 2001, 2002), we presume that lutein, zeaxanthin, and β-cryptoxanthin are carotenoids that parrots acquire directly from food (although some β-cryptoxanthin may also be derived directly from the metabolism of lutein; McGraw et al., 2002). Anhydrolutein and dehydrolutein, however, are rarely reported from plant matter, and instead are thought to be metabolic products of dietary lutein and/or zeaxanthin in animals (reviewed in McGraw et al., 2002). We previously detected 2′ ,3′ -anhydrolutein in the blood of zebra finches (*Taeniopygia guttata*; McGraw et al., 2002) and the blood and yellow-colored feathers of other estrildid finches (K. McGraw and J. Schuetz, unpublished data). 3′ -Dehydrolutein has been reported in the yellow feathers of a few finches (e.g., Pine Grosbeak [*Pinicola enucleator*], family Fringillidae) and other songbirds (e.g., Pekin robin [*Leiothrix lutea*], family Timaliidae) (Stradi, 1998). As parrots are a monophyletic group and distantly related to passerines, it seems that the ability to manufacture these xanthophyll derivatives (presumably in the liver or small intestine; McGraw et al., 2002) has evolved at least twice in birds. Moreover, carotenoid metabolism has frequently been highlighted in the context of sexual selection and trait costliness in birds (Hill, 1996, 2000). However, in a group lacking carotenoid-based integumentary pigmentation, again alternate, physiological functions of these synthetic carotenoids must be investigated.

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