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Author(s): Jocelyn Hudon, Alline Storni, Elena Pini, Marina Anciães, and Riccardo Stradi

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RHODOXANTHIN AS A CHARACTERISTIC KETO-CAROTENOID OF MANAKINS (PIPRIDAE)

JOCELYN HUDON,^{1,4} ALLINE STORNI,^{2,5} ELENA PINI,² MARINA ANCIÃES,³ AND RICCARDO STRADI²

¹Royal Alberta Museum, 12845 – 102 Avenue, Edmonton, Alberta T5N 0M6, Canada;

²Dipartimento di Scienze Molecolari Applicate ai Biosistemi, Sezione di Chimica Organica “A. Marchesini,”
Università degli Studi di Milano, Via Venezian, 21. 20133, Milano, Italy; and

³Coordenação de Biodiversidade e Programa de Coleções e Acervos Científicos, Instituto Nacional de Pesquisas da Amazônia –
INPA, Av. André Araújo, 2936, Aleixo, CEP 69011-970, Caixa Postal 478, Manaus, Amazonas, Brazil

ABSTRACT.—Manakins are small suboscine passerines renowned for elaborate courtship displays and pronounced sexual dichromatism. We extracted and identified the carotenoid pigments found in the bright yellow to red feathers of 15 Neotropical manakin species. Rhodoxanthin (4',5'-didehydro-4,5'-retro- β,β -carotene-3,3'-dione) of a deep red hue was widely found in the plumages of these birds. The 4-keto-carotenoids typically found in red plumages (α -doradexanthin, astaxanthin, canthaxanthin, etc.) were found in only the Flame-crested Manakin (*Heterocercus linteatus*) and the Red-headed Manakin (*Pipra rubrocapilla*). Rhodoxanthin was recently identified in the Pin-tailed Manakin (*Ilicura militaris*). Hudon et al. (2007) suggested that the Pin-tailed Manakin produces rhodoxanthin endogenously from dietary carotenoids, and this suggestion now finds some support in the pigment's wide and specific distribution in manakins. It is possible that this capacity arose in a common ancestor of manakins and cotingas, given that rhodoxanthin also occurs in the feathers of red cotingas (*Phoenicircus* spp.). Feathers with rhodoxanthin absorbed longer wavelengths of light than those with 4-keto-carotenoids, but not in *Pipra*, which has species with one or the other type of keto-carotenoids. We found that feathers with rhodoxanthin also contained ϵ,ϵ -caroten-3(3')-ones (e.g., canary-xanthophyll B and the recently described piprixanthin), which are believed to be intermediates along the rhodoxanthin pathway. Manakins thus have the capability to produce a wide range of carotenoid pigments and bright yellow to red colors, on which natural and sexual selection may act. Received 13 October 2011, accepted 17 April 2012.

Key words: canary-xanthophyll B, carotenoid pigmentation, ϵ,ϵ -caroten-3(3')-ones, plumage, red cotingas, sexual selection.

A rodoxantina como um cetocarotenoide típico de tangarás (Pipridae)

RÉSUMÉ.—Tangarás são pequenos passeriformes suboscíneos famosos pelos seus rituais de cõrte elaborados e pelo dicromatismo sexual acentuado. Nós extraímos e identificamos os pigmentos carotenoides das penas, que vão do amarelo brilhante ao vermelho, de 15 espécies de tangarás neotropicais. A rodoxantina (4',5'-didehidro-4,5'-retro- β,β -carotene-3,3'-dione) com um tom de vermelho profundo foi amplamente encontrada na plumagem dessas aves. Os 4-ceto-carotenoides, tipicamente encontrados em plumagens vermelhas (α -doradexantina, astaxantina, cantaxantina etc.), foram encontrados apenas no *Heterocercus linteatus* e no *Pipra rubrocapilla*. A rodoxantina foi identificada recentemente no *Ilicura militaris*. Hudon et al. (2007) propuseram que *I. militaris* produz rodoxantina de maneira endógena a partir de carotenoides obtidos na dieta; esta proposição encontra agora apoio na distribuição ampla e específica desse pigmento em tangarás. É provável que essa habilidade tenha surgido em um ancestral comum de tangarás e cotingas, já que a rodoxantina também ocorre nas penas de *Phoenicircus* spp. Penas com rodoxantina absorvem comprimentos de onda da luz mais longos do que aquelas com 4-ceto-carotenoides, mas não em *Pipra*, que possui espécies com um ou outro tipo de cetocarotenoide. Observamos que penas com rodoxantina também continham ϵ,ϵ -carote-3(3')-onas (e.g., canário-xantofila B e a recentemente descrita piprixantina), que acredita-se serem intermediárias na via metabólica da rodoxantina. Os tangarás, portanto, têm a capacidade de produzirem uma ampla variedade de pigmentos carotenoides e cores do amarelo brilhante ao vermelho, sobre as quais pode atuar a seleção sexual.

MANAKINS (PIPRIDAE) ARE small, arboreal, frugivorous suboscine passerines endemic to the tropical forests of Central and South America. The adult males in many species have gaudy plumages, often with contrasting patches of brilliant colors like

red or yellow, but also white, blue, and black, that stand in sharp contrast to the largely greenish plumages of females and young birds (Snow 1963, Sick 1967). Male manakins display these bold colors and patterns to females at leks using elaborate dances and

⁴E-mail: jocelyn.hudon@gov.ab.ca

⁵Present address: Foco Ambiental Consultoria, Rua 4, 197B, Cidade Nova, CEP 68615-000, Parauapebas, Pará, Brazil.

courtship displays, and they contribute only sperm to reproduction (Snow 1963, Sick 1967, Prum 1990). This results in high variance in male mating success and intense sexual selection (Skutch 1967; McDonald 1989a, b; Snow 2004; Ryder et al. 2009).

Manakins owe their bold colors to both structural elements and pigmentation in the feathers (Frank 1939, Brush 1969). Of these, carotenoid pigments provide the strongest chromatic, as well as high achromatic, contrasts in the forest-shade environments that many manakins use (Heindl and Winkler 2003, Doucet et al. 2007). Carotenoid displays are of particular interest because they are believed to convey information on the carriers (Hill 1991, Hill and Montgomerie 1994, Lozano 1994, Olson and Owens 1998). Carotenoid-based colors can range from yellow to red, thereby also offering a palette of colors on which natural and sexual selection may act.

Little work has so far been done to characterize the carotenoid pigments of manakins. The only comparative work on the carotenoids of manakins to date has come from a study of the pigments found in the feathers of several members of the *Pipra erythrocephala* superspecies (including the Round-tailed Manakin [*P. chloromeros*]), which exhibit different combinations of yellow, red, and white colors on the head, underwing, and thigh areas (Hudon et al. 1989). As in many birds, the bright yellow color patches in these taxa contain carotenoids commonly found in diets (lutein and zeaxanthin), whereas the bright red patches contain mainly endogenously produced 4-keto carotenoids (e.g., α -doradoxanthin, astaxanthin, and canthaxanthin; Hudon et al. 1989).

Therefore, it was a great surprise when a recent study of another manakin, the Pin-tailed Manakin (*Ilicura militaris*), yielded rhodoxanthin (4',5'-didehydro-4,5'-retro- β,β -carotene-3,3'-dione), a carotenoid of a deep red hue, as its sole red pigment (Hudon et al. 2007). Known from vegetative structures of a variety of mostly non-angiosperm plants and a few fruits (see review in Hudon et al. 2007), rhodoxanthin has been found in few animals, including birds. First isolated and characterized in an animal in the dark red feathers of two species of red cotingas (*Phoenicircus nigricollis* and *Ph. carnifex*; Völker 1951, 1952), rhodoxanthin has since been recorded in birds only in the plumages of several species of fruit-doves (Treroninae) mainly from Indonesian and Pacific archipelagos, notably in the genus *Ptilinopus* (Völker 1953), and in the red feathers of the head of male Western Tanagers (*Piranga ludoviciana*; Hudon 1991). Recently the pigment was implicated in the appearance, in the past 50 years, of orange-colored variants of several species of songbirds, notably the Cedar Waxwing (*Bombycilla cedrorum*), although consumption of berries of exotic bush honeysuckles that contain the pigment is suspected in this case (Hudon and Brush 1989, Brush 1990, Mulvihill et al. 1992, Witmer 1996).

Rhodoxanthin has long been assumed to be acquired exogenously by birds (Völker 1952, Thommen 1971, Hudon 1991), but its absence in a plumage color variant of the Pin-tailed Manakin (Anciães et al. 2005), as well as a logical sequence of chemical transformation linking the manakin's feather pigments to pigments available in the diet, rather implied that it was produced endogenously by that species. The suggestion that an animal might be able to produce rhodoxanthin from dietary carotenoids, though provocative, is not without precedent. Almost 30 years ago, Matsuno

and Katsuyama (Matsuno et al. 1980, Matsuno and Katsuyama 1982, Katsuyama and Matsuno 1988) demonstrated that Nile Tilapia (*Oreochromis niloticus*) maintained their integumentary rhodoxanthin with only supplements of lutein and zeaxanthin.

The Pin-tailed Manakin and a close relative, the Golden-winged Manakin (*Masius chrysopterus*), also display ϵ,ϵ -caroten-3(3')-ones, like canary-xanthophyll B, including one carotenoid new to science, piprixanthin (6-hydroxy- ϵ,ϵ -carotene-3,3'-dione), in their colorful feathers. The ϵ,ϵ -caroten-3(3')-ones absorb at shorter wavelengths than dietary carotenoids (Brockmann and Völker 1934, Hudon 1991) and have been found in the bright yellow and greenish plumages of many passerines (Brockmann and Völker 1934, Hudon 1991, McGraw et al. 2001), though in no other suboscines.

In the present study, we identified the carotenoid pigments found in the feathers of 15 manakin species (of a total of 51) in 8 genera (of a total of 13; Prum 1992, Remsen et al. 2011) using a mild method of chemical extraction and separation (Stradi et al. 1995). Our goals were to (1) evaluate how unusual rhodoxanthin is in manakins and assess the significance of its occurrence in the Pin-tailed Manakin; and (2) identify the carotenoid pigments responsible for the bright yellow to red colors seen in manakins, and associated metabolic activities. Because rhodoxanthin is known to absorb at longer wavelengths than common 4-keto-carotenoids (Kuhn and Brockmann 1933), we also examined, for 18 species with orange to red patches, whether feathers harboring the pigment displayed reflectance spectra that were shifted further into the visible light spectrum.

METHODS

Biological materials.—The bird collection (Coleção Ornitológica) of the Museu de Zoologia da Universidade de São Paulo (Material Transfer Agreement no. 023/2007 MZUSP) donated brightly colored feathers from adult males of 15 species of manakins native to Brazil (Table S1, an online supplement; see Acknowledgments). We included at least one species from all genera of Brazilian manakins with red in their plumages (11 species in six genera), as well as several taxa with bright yellow color patches (four species in two genera; Table 1). For two species with both red and yellow displays, we sampled feathers from each color (Table 1). For pigment analyses, we pooled feathers of a particular patch color from all individuals of a given species (~5 feathers) because only one or two, rarely three, feathers were made available from each sampled specimen to minimize specimen destruction.

Extraction procedure.—We extracted and concentrated feather carotenoids as follows (modified from Stradi et al. 1995): we carefully washed ~3 mg of colored barbs with hexane on a glass filter and finely ground the barbs in the presence of 2 mL of methanol and 25 zirconium oxide balls in a ZrO container with a Retsch MM301 mixer mill (Hann, Germany) for 10 min at 900 rpm at room temperature. We completely removed the solid residues (inorganic salts and feather proteins) from the solution containing carotenoids through filtration on Sep-Pak C₁₈ cartridges (Waters Millipore, Milford, Massachusetts). We evaporated the filtrate containing the carotenoid pigments under a stream of dry N₂ and dissolved the residue in the high-performance liquid chromatography (HPLC) mobile phase.

TABLE 1. Relative proportions of the principal carotenoids found in the manakins analyzed. H = 6-hydroxy- ϵ,ϵ -carotene-3,3'-dione B = ϵ,ϵ -carotene-3,3'-dione, L = lutein, Z = zeaxanthin, D = α -doradoxanthin, E = 3-hydroxy-echinenone, C = canthaxanthin, P = adonirubin, A = astaxanthin, R + r = rhodoxanthin, and n.d. = not detectable. Proportions are from the pooling of feathers from several individuals and are, thus, representative only.

Species	Body part (color)	Percentage of total absorption at 450 nm by the principal carotenoids (peak of absorption in methanol [nm])									
		H (438)	B (438)	L (445)	Z (450)	D (450)	E (455)	C (466)	P (468)	A (468)	R+r (490)
Pale-bellied Tyrant-Manakin (<i>Neopelma pallescens</i>)	Forehead (yellow)	n.d.	<1	53	36	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pin-tailed Manakin (<i>Ilicura militaris</i>)	Forehead (red)	2	24	5	7	n.d.	n.d.	n.d.	n.d.	n.d.	52
Eastern Striped Manakin (<i>Machaeropterus regulus</i>)	Forehead (red)	2	15	11	8	n.d.	n.d.	n.d.	n.d.	n.d.	56
Blue-crowned Manakin (<i>Lepidothrix coronata</i>)	Breast (yellow)	n.d.	<1	22	63	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Snow-capped Manakin (<i>L. nattereri</i>)	Breast (yellow)	n.d.	<1	61	24	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
White-fronted Manakin (<i>L. serena</i>)	Breast (yellow)	n.d.	<1	81	9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Helmeted Manakin (<i>Antilophia galeata</i>)	Head (red)	5	30	<1	<1	n.d.	n.d.	n.d.	n.d.	n.d.	64
Blue-backed Manakin (<i>Chiroxiphia pareola</i>)	Head (red)	6	51	<1	<1	n.d.	n.d.	n.d.	n.d.	n.d.	45
Swallow-tailed Manakin (<i>C. caudata</i>)	Head (orange)	5	62	<1	<1	n.d.	n.d.	n.d.	n.d.	n.d.	26
Flame-crested Manakin (<i>Heterocercus linteatus</i>)	Crown (red)	n.d.	n.d.	n.d.	45	14	3	n.d.	n.d.	37	n.d.
Crimson-hooded Manakin (<i>Pipra aureola</i>)	Head (red)	2	2	20	5	n.d.	n.d.	n.d.	n.d.	n.d.	65
	Breast (red)	2	2	32	16	n.d.	n.d.	n.d.	n.d.	n.d.	45
	Neck (yellow)	n.d.	<1	63	27	n.d.	n.d.	n.d.	n.d.	n.d.	<1
Wire-tailed Manakin (<i>P. filicauda</i>)	Head (red)	1	6	30	6	n.d.	n.d.	n.d.	n.d.	n.d.	53
	Breast (yellow)	n.d.	<1	67	13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Band-tailed Manakin (<i>P. fasciicauda</i>)	Head and breast (red)	2	5	19	16	n.d.	n.d.	n.d.	n.d.	n.d.	51
Golden-headed Manakin (<i>P. erythrocephala</i>)	Head (yellow)	n.d.	<1	75	11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Red-headed Manakin (<i>P. rubrocapilla</i>)	Head (red)	n.d.	n.d.	n.d.	10	41	5	11	3	29	n.d.

Apparatus and HPLC-UV-vis-MS analyses.—We used a Thermo Finnigan instrument LCQ Advantage Series HPLC (Thermo Finnigan, San Jose, California) equipped with a Surveyor quaternary pump, a diode-array detector, an electrospray ionization source (ESI), and an ion trap analyzer (positive mode) for liquid chromatography–mass spectrometry (LC–MS) analyses, and to obtain ultraviolet (UV)-visible and mass spectra of eluted carotenoids. We carried out carotenoid separations using a YMC-pack C30 column (5 mm; 250 × 4.6 mm I.D.; YMC, Kyoto, Japan). We used a methanol/*tert*-butyl methyl ether (95/5, v/v) mobile phase running at 0.8 mL min⁻¹ to separate the carotenoids in all samples. We injected the samples using a Rheodyne (model 7125) valve equipped with a 20- μ L loop.

We acquired spectral data for the wavelength range 230–600 nm, using a Surveyor photodiode-array detector, and integrated the areas under the peaks detected at 450 nm. We recorded three-dimensional chromatograms using Totalchrom Workstation software (Perkin-Elmer). The MS parameters were as follows: capillary voltage, 5 V; capillary temperature, 250°C;

dry gas N₂; flow, 1L h⁻¹; the mass spectra were acquired with a scan range of m/z from 300 to 700. We identified the carotenoids on the basis of their visible spectra, mass on mass spectrometer, and co-chromatography with known standards. Because of drift between runs, only peaks with good mass spectra were identified and quantified.

Chemicals and reagents.—We obtained methanol and *tert*-butyl methyl ether from Sigma-Aldrich (Switzerland). Hoffman-LaRoche (Basel, Switzerland) kindly supplied standards of ϵ,ϵ -carotene-3,3'-dione and lutein. CaroteNature (Lupsingen, Switzerland) provided standards of zeaxanthin and rhodoxanthin.

Spectrophotometry.—M.A. acquired UV-visible reflectance spectra across the visible range of birds (Goldsmith 1990) of bright yellow to red patches in a wide range of manakins. The specimens were different from those that we characterized biochemically, selected from skins made available by various museums (listed in Acknowledgments). Reflectance spectra obtained from museum specimens are no different from those obtained from live representatives of these species (M. Anciães unpubl. data).

M.A. obtained reflectance spectra using an Ocean Optics USB2000 spectrophotometer (Ocean Optics, Dunedin, Florida), operated with OOIBASE32, version 2.0.1.4, attached to a PX-2 Xenon Pulsed light source, after standardization with a 97% reflection white standard (Labsphere, North Sutton, New Hampshire). She took readings (5 averaged) from standard locations on the plumage of each bird, and additionally from unique color patches in a species-specific manner. She acquired spectra at a standard 6-mm distance from the plumage patch, placing the probe directly above the patch, because the colors were solid (i.e., not shiny) and there was no need to control for glossiness or iridescence (see Endler 1990). M.A. took spectral readings at 0.40-nm intervals from 300 nm to 700 nm, and interpolated to 1-nm intervals for subsequent analysis.

The hue of plumage patches that harbor carotenoids is controlled largely by the position of the slope of increasing reflectance at longer wavelengths (Montgomerie 2006, Andersson et al. 2007), which can be assessed by calculating the λ_{R50} of the reflectance spectrum, the wavelength where reflectance is at the midpoint between the maximum reflectance at long wavelengths and the minimum at shorter wavelengths. We used the software CLR, version 1.05 (Montgomerie 2008), to calculate λ_{R50} values of the different color patches scanned. We used a program developed by Rafael Maia (available upon request; see Acknowledgments) to emulate TETRACOLORSPACE, version 1a, of Stoddard and Prum (2008) in the R environment (R Development Core Team 2006) to calculate hue θ , a metric from a more complex color system developed to describe colors in a tetrahedral color space that takes into account the visual system of birds (e.g., Goldsmith 1990, Stoddard and Prum 2008). We note that λ_{R50} values correlated well with hue θ (Spearman rank $r_s = -0.934$, $P < 0.0001$, $n = 29$). However, because λ_{R50} values are much easier to visualize than hue θ , while capturing the spectral features of interest, we report and discuss only λ_{R50} here.

RESULTS

Pigmentation.—In total, we identified and quantified at least 10 carotenoid pigments in the feathers of the manakins analyzed (Table 1). The yellow feathers of species with only bright yellow colors (four species in two genera) as well as those with some red in their plumages (three species in another genus) consistently yielded the common hydroxy-carotenoids zeaxanthin and lutein almost exclusively (Fig. 1A and Table 1).

Compared with yellow feathers, the orange to red feathers from 10 species in 6 genera contained one or more red keto-carotenoids, as well as several additional yellow carotenoids (Fig. 1B and Table 1).

Surprisingly, the manakins examined fell into two groups with respect to the type of red keto-carotenoid(s) they deposited in their feathers; no taxa showed both types concurrently. Two species of manakins (*Pipra rubrocapilla* and *Heterocercus linteatatus*) harbored several common 4-keto-carotenoids (astaxanthin, adonirubin, canthaxanthin, and α -doradexanthin) in their feathers, along with zeaxanthin but no lutein. The vast majority of species with red in their plumages, however, instead harbored several stereoisomers of rhodoxanthin (Table 1), a retro-carotenoid of deep red hue. Three isomers (6-*trans*, 6'-*trans* [R]; 6-*trans*, 6'-*cis*

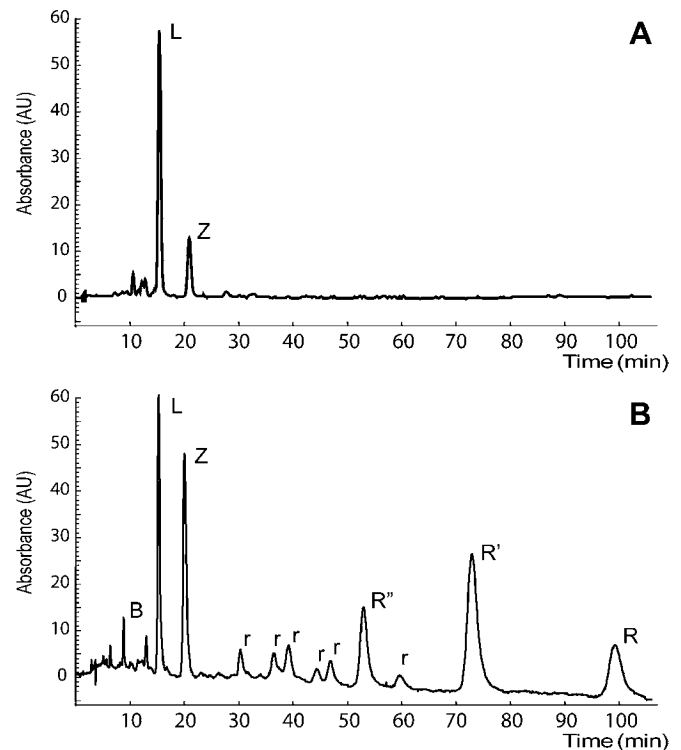


FIG. 1. (A) Typical high-performance liquid chromatography (HPLC) chromatogram of carotenoids present in yellow feathers of adult male manakins. This one was produced using the yellow feathers on the head of *Pipra erythrocephala* specimens MZUSP 60030, MZUSP 17780, and MZUSP 78979 (MZUSP = Museu de Zoologia da Universidade Federal de São Paulo, Brazil). (B) Typical HPLC chromatogram of carotenoids present in orange-red feathers of adult male manakins: red head feathers of *P. aureola* specimens MZUSP 60025, MZUSP 60022, and MZUSP 60024. Carotenoids were eluted using a mobile phase of methanol/MTBE (95/05, v/v) running at 0.8 mL min⁻¹. See Table 1 for carotenoid associated with each letter.

or 6-*cis*, 6'-*trans* [R']; and 6-*cis*, 6'-*cis* [R'']), based on λ_{max} values of 500 nm, 496 nm, and 492 nm in the mobile phase, respectively) were the most abundant (see Fig. 1B), but several peaks with the same molecular weight as rhodoxanthin (562.84 D) and UV-visible spectra consistent with a *cis*-configuration of the polyunsaturated chain of double bonds were also present (labeled "r" on Fig. 1B). Their exact stereo-structure was not elucidated further.

Interestingly, the feathers that harbored rhodoxanthin (but not those that harbored 4-keto-carotenoids) also contained several yellow pigments with shortened polyene central chains that absorb maximally at shorter wavelengths than common dietary carotenoids (lutein and zeaxanthin), which were not apparent in the yellow feathers. These yellow carotenoids included ϵ,ϵ -carotene-3,3'-dione (also known as canary-xanthophyll B) and 6-hydroxy- ϵ,ϵ -carotene-3,3'-dione, a novel carotenoid first identified in the Pin-tailed Manakin and named "piprixanthin" (Hudon et al. 2007). The latter pigment has been suggested as a direct precursor of rhodoxanthin (Hudon et al. 2007). Red feathers from three species of manakins with rhodoxanthin—the Helmeted Manakin (*Antilophia galeata*), Swallow-tailed Manakin

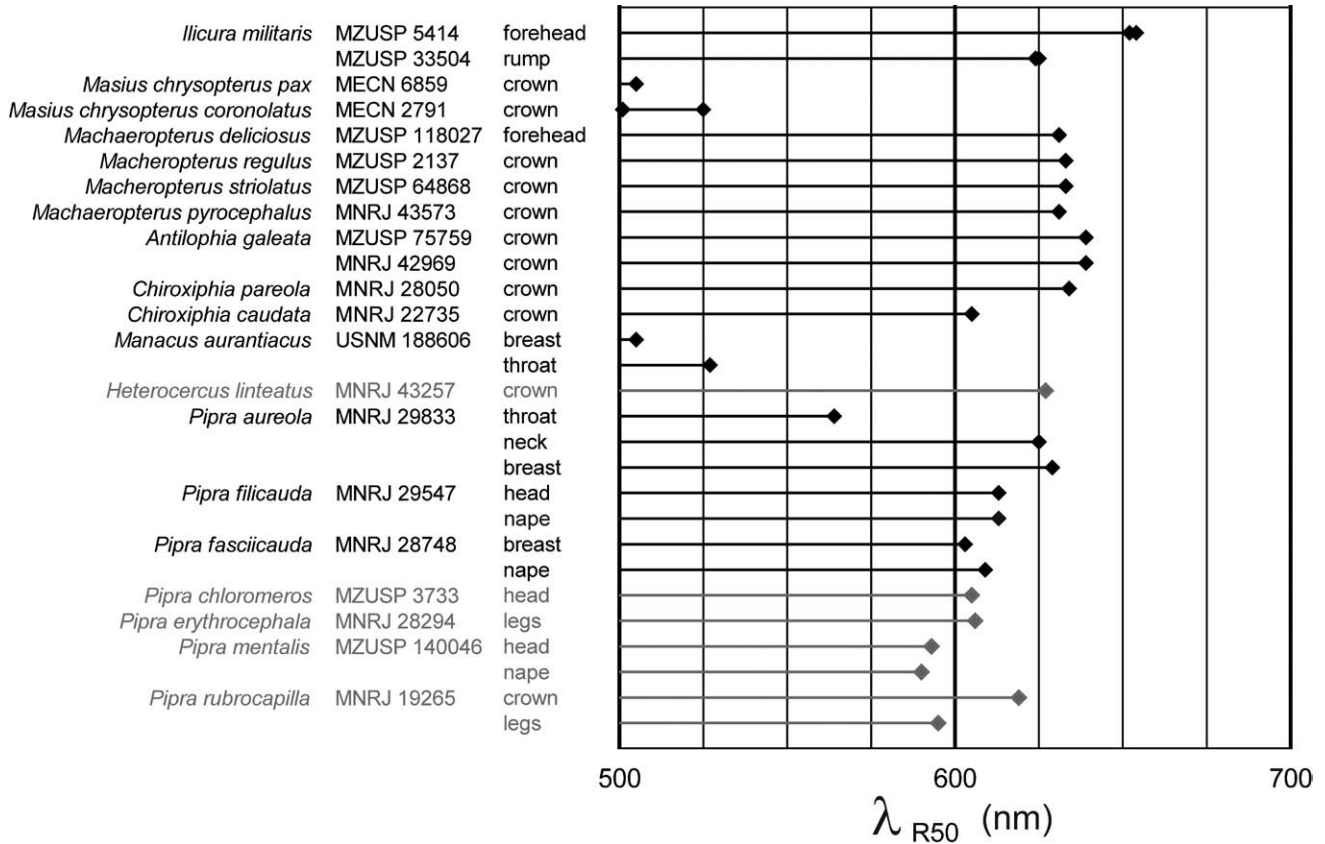


FIG. 2. The λ_{R50} values (diamonds) of plumage patches of orange to red color in several manakins with such colors. Those species known or suspected to display rhodoxanthin are identified in black; those that display 4-keto-carotenoids are in gray. The keto-carotenoids in *Manacus aurantiacus* are not known; they are possibly 4-keto-carotenoids. Species sequence follows Prum (1992) and Remsen et al. (2011). Abbreviations: MZUSP = Museu de Zoologia da Universidade Federal de São Paulo, Brazil; MNRJ = Museu Nacional da Universidade Federal do Rio de Janeiro; MECN = Museo Ecuatoriano de Ciencias Naturales; and USNM = U.S. National Museum, Smithsonian Institution, Washington, D.C.

(*Chiroxiphia caudata*), and Blue-backed Manakin (*C. pareola*)—lacked the dietary carotenoids lutein and zeaxanthin altogether (Table 1).

Spectrophotometry.—The feathers that harbored rhodoxanthin tended to have spectra that were shifted further into the green part of the spectrum (higher λ_{R50}) than those bearing 4-keto-carotenoids (Fig. 2), although not within the genus *Pipra*, which had representatives using one or the other type of keto-carotenoid (Fig. 2). We recorded the highest λ_{R50} values for the forehead feathers of the Pin-tailed Manakin. With λ_{R50} values of 652 to 654 nm, these feathers had spectra that were shifted ~25 nm further into the green part of the visible spectrum than the crown feathers of the Flame-crested Manakin (*Heterocercus linteatus*; 627 nm), the feathers bearing 4-keto-carotenoids with the highest recorded λ_{R50} value (Fig. 2).

DISCUSSION

Far from being a singularity in the Pin-tailed Manakin, rhodoxanthin is widely distributed in the red to orange feathers of manakins. Nine of 11 taxa examined with red tints in their plumages, including species from all genera with red in their plumages

except one (*Heterocercus*), had rhodoxanthin as their sole red keto-carotenoid. The wide distribution of rhodoxanthin in manakins lends added weight to the suggestion made by Hudon et al. (2007), mainly on the basis of the pigments in a rare color variant, that the pigment is produced endogenously by members of this family. Indeed, it would be surprising for many taxa in particular genera of manakins to be able to reliably acquire the pigment exogenously in the wild, given its relative scarcity in fruits in nature (although the carotenoid make-up of tropical fruits eaten by manakins is still largely unknown). If manakins can produce rhodoxanthin endogenously from dietary carotenoids, that capability was already well established in the ancestors of manakins, judging from the presence of this pigment in lineages that split early in the evolution of the group (Tello et al. 2009, McKay et al. 2010).

The presence of rhodoxanthin in the red cotingas suggest that the capability possibly evolved even earlier than that, possibly pre-dating the split of cotingas and manakins. Although cotingas and manakins have long been regarded as each other's closest relatives (Snow 2004, Ericson et al. 2006), recent molecular work suggests that their relationships may be more distant. In two recent studies, cotingas were found to be sister taxa to a clade that comprises *Pachyramphus*, *Tytira*, *Schiffornis*, *Laniisoma*, and *Oxyruncus*,

together the sister taxa to the clade of tyrant-flycatchers, with the manakins falling outside this large group (Johansson et al. 2002, Tello et al. 2009). Thus, the capability to produce rhodoxanthin endogenously could conceivably have evolved quite early, before the large radiation of New World suboscines. Although rhodoxanthin has not been described in suboscines outside manakins and cotingas, a systematic study of their carotenoid pigments is still largely lacking (none listed in McGraw 2006).

Alternatively, red cotingas and manakins are subject to similar selection pressures, and converged in appearance and types of pigments deposited through the evolution of the capability to produce rhodoxanthin on possibly two separate occasions. We note that the red cotingas share with manakins many aspects of courtship, vocalizations, and morphology (e.g., short wings and tail, intense carotenoid-rich plumage and foot structure; Trail and Donahue 1991) and, in fact, look like large versions of manakins. It is not clear why rhodoxanthin has so far not been found in any cotingid other than the red cotingas, despite the good number of cotingas that have had carotenoids characterized (see Völker 1952, Brush 1969, Thommen 1971, LaFountain et al. 2010).

Curiously, the 4-keto-carotenoids that are characteristically associated with bright red to orange feathers in other birds (Thommen 1971, Brush 1981, Hudon 1991, Stradi 1998, McGraw 2006) occurred in only two of the taxa examined here, the Red-headed Manakin (*Pipra rubrocapilla*) and the Flame-crested Manakin. The pigments were previously recorded in the Red-headed Manakin and two close cousins, the Golden-headed Manakin (*P. erythrocephala*) and the Round-tailed Manakin, and taken to be the norm in this family at the time (Hudon et al. 1989), though they now appear to be the exception. The Red-headed Manakin, Golden-headed Manakin, and Red-capped Manakin (*P. mentalis*) constitute a superspecies (Sibley and Monroe 1990), to which the Round-tailed Manakin is sometimes added (Snow 1979). These species, together with the Scarlet-horned Manakin (*P. cornuta*), are sometimes placed in a separate subgenus (*Ceratopipra*) within *Pipra* (Prum 1992). *Heterocercus* is believed to be closely related to *Pipra* (Prum 1992), although recent molecular work has placed them more distantly (McKay et al. 2010). Thus, not only are 4-keto-carotenoids sparsely distributed in manakins, they are found in only a few clades within the family. Only the manakin genus *Manacus* has a species with reddish tones, the Orange-collared Manakin (*Manacus aurantiacus*), whose feathers were not available for examination. We believe that the capability to produce 4-keto-carotenoids is ancestral in manakins, given that it is widely present in passerines and nonpasserines (Thommen 1971, Stradi 1998, McGraw 2006), and that the capability to produce rhodoxanthin is a later addition.

The question is then why birds that already had the ability to synthesize red keto-carotenoids from dietary carotenoids (the widely distributed 4-keto-carotenoids) would evolve another such pigment (rhodoxanthin). One possible answer is that, because rhodoxanthin absorbs maximally at longer wavelengths than 4-keto-carotenoids, it has the potential to produce colors that are shifted further into the green part of the spectrum than 4-keto-carotenoids. Some of these colors would look darker red to purple to our eyes (as their hue θ of -0.51 falls between the vertices for pure blue [2.618] and pure red colors [0.52] on the bird tetrahedral color space; Goldsmith 1990, Stoddard and Prum 2008; data not

shown) and could have been favored by strong sexual selection for innovations (Stoddard and Prum 2008, LaFountain et al. 2010). Support for the view that rhodoxanthin allows birds to expand into new color territories comes from the observation that the highest recorded λ_{R50} values in manakin feathers were those that contained rhodoxanthin as their keto-carotenoid. Although an increase in pigment concentration can also lead to a shift to longer wavelengths and higher λ_{R50} values (Hudon et al. 2003, Anderson et al. 2007), no manakin with 4-keto-carotenoids had color patches that were as red-shifted as those displaying rhodoxanthin.

In fruit-doves, rhodoxanthin produces feather colors that range widely, from red to blue, through purple-red and violet. It is believed that the color change results, in this case, from the interaction of the carotenoid with feather proteins that shift the pigment's absorption spectrum to even longer wavelengths, which can be mimicked in solution by exposing rhodoxanthin to various adsorption media (e.g., silica, calcium carbonate, aluminum oxide, and talc) or solvents (Völker 1953). In other birds these colors are produced only through unique pigments or structures (Görnitz and Rensch 1924, Auber 1957, LaFountain et al. 2010).

But the shift to longer wavelengths in manakins was not large, of the order of 25 nm, and was even absent in some species. We note for example that the λ_{R50} values for red patches of *Pipra* species bearing rhodoxanthin were not much different from those of congeners bearing 4-keto-carotenoids. Also, some feathers with rhodoxanthin were orange in hue (e.g., in the Swallow-tailed Manakin) rather than red and, thus, do not require a carotenoid of such deep red hue. However, it is probable that these feathers would exhibit a stronger bathochromic shift (absorption at longer wavelengths) were it not for the presence of substantial amounts of yellow ϵ,ϵ -caroten-3(3')-ones, like canary-xanthophyll B, in all feathers that contained rhodoxanthin. The ϵ,ϵ -caroten-3(3')-ones absorb at shorter wavelengths than common dietary carotenoids and, thus, have the potential to counterbalance the shift to longer wavelengths that rhodoxanthin brings about. This counteracting action would generate hues not unlike those produced by 4-keto-carotenoids, at least to the human eye, which might help explain why the presence of rhodoxanthin has gone unnoticed in manakins to this day. Manakin feathers with 4-keto-carotenoids lacked the yellow ϵ,ϵ -caroten-3(3')-ones.

Yellow ϵ,ϵ -caroten-3(3')-ones, like canary-xanthophyll B, are perhaps not surprising in feathers that bear rhodoxanthin, because they have been hypothesized to be direct intermediates on the path from zeaxanthin to the keto-carotenoid in the Pintailed Manakin (Hudon et al. 2007; Fig. 3), with a newly described carotenoid, piprixanthin, forming a bridge between the shorter-wavelength-absorbing ϵ,ϵ -caroten-3(3')-ones and rhodoxanthin (Hudon et al. 2007). Possibly the ϵ,ϵ -caroten-3(3')-ones are residuals of the biochemical processes that produce rhodoxanthin from dietary carotenoids. That is not to say that yellow ϵ,ϵ -caroten-3(3')-ones always need to accompany rhodoxanthin in feathers, only that they did in all taxa we studied that had the keto-carotenoid. We note that the conditions that led to the evolution of the biochemical pathway may be very different from those that result in its use in modern manakins. Ironically, the evolutionary step to a deeper red pigment in birds appears to have been through production of yellow pigments that absorb at shorter wavelengths than the yellow pigments present in the diet.

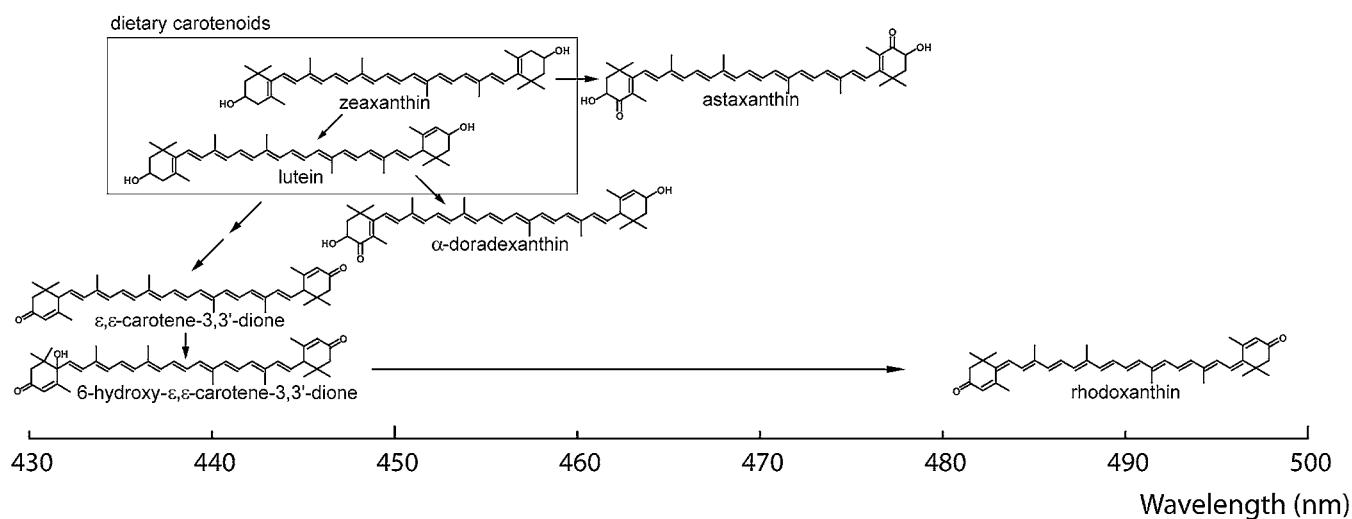


FIG. 3. Presumed pathways of formation of red keto-carotenoids from common dietary carotenoids (zeaxanthin and lutein) in manakins, superimposed on a section of the scale of visible wavelengths. The carotenoids are broadly centered above the wavelength of their peak absorption.

Finally, we note that, although rhodoxanthin and 4-keto-carotenoids may be largely equivalent to produce red colors, none of the studied taxa with rhodoxanthin in their feathers also displayed 4-keto-carotenoids (and vice versa). The only exception that we know of is a specimen of the Round-tailed Manakin studied previously that harbored both 4-keto-carotenoids and rhodoxanthin. However, because rhodoxanthin was present in small amounts, alongside more abundant 4-keto-carotenoids, at the time it was suggested that the retro-carotenoid might have been acquired exogenously by the individual (Hudon et al. 1989). In light of the hypothesized ability of manakins to produce rhodoxanthin endogenously and the possibility of reversals and activation of silent biochemical pathways, perhaps this conclusion needs to be reassessed.

Segregation of the two types of keto-carotenoids is not unexpected when we consider that they lie on largely divergent, possibly competing biochemical pathways, starting with carotenoids available in the diet (lutein and zeaxanthin; McGraw et al. 2003; also see Fig. 3). Indeed, whereas production of 4-keto-carotenoids involves the addition of carbonyl groups directly on carbon-4(4') of the β -end-rings of dietary carotenoids, the production of rhodoxanthin involves the move of one or two double bonds (starting with lutein and zeaxanthin, respectively) out of conjugation with the central chain of polyunsaturations, resulting in pigments with ϵ -end-rings that absorb at shorter wavelengths than dietary carotenoids (e.g., canary-xanthophyll B; Matsuno et al. 1985, 1986; Tyczkowski et al. 1986). Once an end-ring takes an ϵ configuration, it is no longer capable of accepting a carbonyl group at carbon 4(4'), unless it reverts back to a β configuration. Thus, production of 4-keto-carotenoids in feathers that manufacture canary-xanthophylls would necessitate halting, at the very least greatly reducing, production of ϵ, ϵ -caroten-3(3')-ones, so that oxygenation at C-4(4') can take place (Fig. 3). The near absence of common dietary carotenoids (zeaxanthin and lutein) in the red feathers of the Helmeted Manakin,

Swallow-tailed Manakin, and Blue-backed Manakin suggests that manakins can convert both zeaxanthin (Hudon et al. 2007) and lutein to rhodoxanthin (see Fig. 3).

Manakins as an aggregate thus have the capability to produce a wide range of carotenoid pigments, and consequently a wide range of bright yellow to red colors, starting with carotenoids acquired in the diet, representing a malleable system on which natural and sexual selection may act. The potential for divergence in color would appear to be greatest in species that produce rhodoxanthin, because they would, at least potentially, be capable of producing both pigments that absorb at shorter wavelengths than common dietary carotenoids (the ϵ, ϵ -caroten-3[3']-ones) and pigments that absorb at longer wavelengths (rhodoxanthin), even compared with 4-keto-carotenoids. Investigating what factors determine which potentialities are actually realized will be promising areas of study in the future.

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