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ORIGINAL PAPER

Molecular diversity, metabolic transformation, and evolution of carotenoid feather pigments in cotingas (Aves: Cotingidae)

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Abstract Carotenoid pigments were extracted from 29 feather patches from 25 species of cotingas (Cotingidae) representing all lineages of the family with carotenoid plumage coloration. Using high-performance liquid chromatography (HPLC), mass spectrometry, chemical analysis, and ^{1}H-NMR, 16 different carotenoid molecules were documented in the plumages of the cotinga family. These included common dietary xanthophylls (lutein and zeaxanthin), canary xanthophylls A and B, four well known and broadly distributed avian ketocarotenoids (canthaxanthin, astaxanthin, α-doradexanthin, and adonixanthin), rhodoxanthin, and seven 4-methoxy-ketocarotenoids. Methoxy-ketocarotenoids were found in 12 species within seven cotinga genera, including a new, previously undescribed molecule isolated from the Andean Cock-of-the-Rock *Rupicola peruviana,* 3′-hydroxy-3-methoxy-β,β-carotene-4-one, which we name rupicolin. The diversity of cotinga plumage carotenoid pigments is hypothesized to be derived via four metabolic pathways from lutein, zeaxanthin, β-cryptoxanthin, and β-carotene. All metabolic transformations within the four pathways can be described by six or seven different enzymatic reactions. Three of these reactions are shared among three precursor pathways and are responsible for eight different metabolically derived carotenoid molecules. The function of cotinga plumage carotenoid diversity was analyzed with reflectance spectrophotometry of plumage patches and a tetrahedral model of avian color visual perception. The evolutionary history of the origin of this diversity is analyzed phylogenetically. The color space analyses document that the evolutionarily derived metabolic modifications of dietary xanthophylls have resulted in the creation of distinctive orange-red and purple visual colors.

Keywords Plumage coloration · Color space modeling · Phylogeny

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Introduction

Many different lineages of birds have evolved the capacity to deposit exogenous, or diet-derived, carotenoids into the

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β-keratin of the feathers as pigments. Some have also evolved the capacity to metabolically transform various dietary carotenoids into new molecules that result in distinctive plumage colors. Recently, McGraw (2006) reported a total 24 different carotenoid molecules that have been biochemically characterized from the integument of $\sim$150 species of birds over the last 90 years. The vast majority of this molecular diversity is the result of metabolic modifications of a relatively few carotenoids that are common in avian diets, including lutein, zeaxanthin, β-cryptoxanthin, and β-carotene. Recent analyses of avian perception of plumage color diversity has shown that both the capacity to deposit and to metabolically modify dietary carotenoid pigments in feathers has greatly broadened the achieved gamut of plumage color of birds (i.e. the realized plumage color diversity) (Stoddard and Prum 2011).

In our recent analysis of the carotenoid pigment composition of the burgundy-purple feathers of the male Pompadour Cotinga *Xipholena punicea* (Cotingidae) (Fig. 1), we described six novel molecules of methoxy-ketocarotenoids (LaFountain et al. 2010). These carotenoids are metabolically derived from dietary precursors having either a β-carotene (β,β-ring) or α-carotene

Fig. 1 Carotenoid plumages of a sample of cotinga species analyzed. **a** Male Black-and-gold Cotinga *Tijuca atra* with lutein yellow wing patch; **b** Male Golden-breasted Fruiteater *Pipreola aureopectus* with lutein and zeaxanthin yellow breast. **c** Male Guianan Cock-of-the-Rock *Rupicola rupicola* with bright orange plumage pigmented by lutein and three ketocarotenoids. **d** Male Purple-throated Fruitcrow *Querula purpurata* with burgundy throat patch pigmented by a mixture of four methoxy-ketocarotenoids. **e** Male Pompadour Cotinga *Xipholena punicea* with burgundy body plumage pigmented by a mixture of eight carotenoids. **f** Male Lovely Cotinga *Cotinga amabilis* with blue structural color and purple throat and belly patches pigmented by cotingin. Photos reproduced with permission of Nick Athanas (**a**, **b**, **c**), Richard O. Prum (**d**), Tanguy Deville (**e**), and Ciro Albano (**f**)

(β,ε-ring) structure. Each molecule is characterized by a carbonyl in the C4 position (i.e. carbon 4 in the six carbon ring), and a methoxyl group in the C3 position within one or both β-rings. Three of these molecules have a double bond between C2 and C3 within one or both of the methoxy-keto-β-rings. The most similar previously described molecules are purple 3-methoxy-carotenoids from marine sponges (e.g. aaptopurpurin and clathriaxanthin; Fattorusso et al. 1992; Matsuno 2001; Liaaen-Jensen et al. 1982), and 3-methoxy-zeaxanthin from the human retinal macula (Bhosale et al. 2007). The methoxy-ketocarotenoids of *Xipholena* appear to be produced metabolically through two novel enzymatic reactions that are previously unknown in birds—O-methylation of a 3-hydroxyl group adjacent to a 4-carbonyl and didehydrogenation of the C2–C3 bond (LaFountain et al. 2010).

The metabolic pathways of carotenoid modification in birds have been characterized by a few controlled dietary experiments and radioactive labeling studies, but the actual enzymes involved remain unknown. Only a few papers have compared carotenoid metabolism among related species to document the evolution of the metabolic pathways contributing to variation in plumage coloration. For example, McGraw and Schuetz (2004) examined the occurrence of 4-ketocarotenoids in feathers and blood of six species of estrildid finches, and concluded that the capacity to metabolically modify dietary xanthophylls into ketocarotenoids was primitive to the estrildid family and had been evolutionarily lost in at least one species of the tribe Poephilini. Little is known about how the evolution of metabolic modification of carotenoids influences the evolution of plumage color diversity.

The discovery of novel, metabolically modified carotenoids in the plumage of *X. punicea* raises several interesting questions: Do any other cotingas make and use these pigments in their plumages? Where in the phylogeny of the cotinga family did the metabolic capacity to make and deposit C3 methoxy-ketocarotenoids in feathers evolve? What is the relationship between molecular diversity and color diversity, as perceived by the birds themselves? The cotingas are a family of $\sim$70 species of Neotropical suboscine passerine birds that vary tremendously in sexual dimorphism, body size, and plumage color (Snow 1982, 2004). Most cotingas are obligatory frugivores (Snow 1982, 2004), which is a diet that is rich in carotenoids (Goodwin 1980). Many cotingas have polygynous breeding systems with female-only parental care and male courtship display. Some polygynous cotingas are strongly sexually dimorphic with brilliant carotenoid plumages (Fig. 1), while others may be sexually monomorphic, drab, and relying on vocal advertisement (e.g. most *Lipaugus* pihas) (Snow 1982, 2004). A few genera of cotingas are monogamous with biparental care and reduced sexual dimorphism, but may still exhibit carotenoid plumages. In total, carotenoid plumages are present in 42 species in 17 genera of cotingas.

Here, we investigate the diversity of the plumage carotenoids in the cotingas. We isolated, analyzed, and identified the carotenoid pigments from feathers of 25 different species of cotingas with yellow, orange, red, pink, or purple plumage coloration. This sample represents between 1 and 4 species from each of the 17 genera of cotingas with obvious carotenoid plumage coloration. We integrate the information on cotinga feather carotenoid diversity with analyses of carotenoid metabolism, modeling of tetrachromatic visual perception, and evolution in a phylogenetic context. We hypothesized metabolic pathways for the production of these molecules from dietary xanthophylls, and identify the enzymatic reactions required for these pathways. We model the visual appearance of these molecularly diverse carotenoid plumages using the tetrachromatic model of avian color perception. The goal is to relate the variation in carotenoid composition to the visual color stimuli perceived by the birds themselves. The result is an integrative perspective on the dynamic evolutionary radiation in carotenoid utilization, physiology, and plumage coloration. By integrating analyses of carotenoid biochemistry, physiology, light spectrophotometry, and color vision physiology, we can begin to understand how social and sexual selection on the visual appearance of plumage coloration has resulted in evolution of diverse novelties in avian carotenoid physiology.

Materials and methods

Feather specimens

Feathers were collected from museum specimens of 25 different species of cotinga (Aves, Cotingidae) including samples from every genus in which there are yellow, orange, red, purple, or pink feather patches (Table 1). *Rupicola peruviana* and *Pyroderus scutatus* were represented by two distinctly plumaged and geographically isolated subspecies. *Pipreola formosa* was represented by two distinctly colored plumage patches for a total of 29 plumage patches. Most specimens sampled were male, but in *Procnias* bellbirds carotenoid plumages are only present in females. In some species, it was not possible to sample enough feathers from rare museum study skins to get enough isolated carotenoids for baseline chromatographic separation of multiple molecules. Therefore, in several of these instances, feathers were collected from museum spirit specimens that had been fixed in 10 % formalin and

Table 1 Species, specimen number, plumage patch, color, and sex of cotinga specimens

Species	Specimen	Sample area	Color	Sex
Carpornis cucullatus	YPM 11140, AMNH 315611	Belly	Yellow	Male
Snowornis subalaris	AMNH 179515	Underwing coverts	Yellow	Male
Phibalura flavirostris	AMNH 494416	Belly	Yellow	Female
Ampelion rufaxilla	YPM 26972	Belly	Yellow	Male
Ampeliodes tschudii	AMNH 181517	Undertail coverts	Yellow	Male
Tijuca atra	AMNH 818775	Secondaries	Yellow	Male
Pipreola aureopectus	YPM 40970, AMNH 155775	Belly	Yellow	Male
Pipreola formosa	AMNH 494478	Belly	Yellow	Male
Pipreola formosa	AMNH 494478	Throat	Orange	Male
Pipreola chlorolepidota	AMNH 494495	Throat	Orange	Male
Pipreola whitelyi	AMNH 305631	Breast	Orange	Male
Procnias tricarunculata	YPM 14873	Belly	Yellow	Female
Pyroderus scutatus scutatus	YPM 66958	Throat	Orange	Male
Pyroderus scutatus occidentalis	AMNH 494846	Throat	Orange	Male
Phoenicircus carnifex	KU 88704	Belly	Red	Male
Rupicola rupicola	YPM 24943, 84425	Belly	Orange	Male
Rupicola peruviana saturata	YPM 81787	Belly	Orange	Male
Rupicola peruviana sanguinolenta	YPM 1147	Back and belly	Red	Male
Xipholena punicea	YPM 84426	Back and belly	Burgundy	Male
Xipholena lamellipennis	YPM 304, AMNH 78264	Back	Black	Male
Xipholena atropurpurea	AMNH 494643	Back	Black	Male
Haematoderus militaris	KU 88718	Back and belly	Crimson	Male
Querula purpurata	YPM 137213	Throat	Crimson	Male
Cotinga cotinga	YPM 15320	Breast	Purple	Male
Cotinga maculata	AMNH 11030	Breast	Purple	Male
Cotinga amabilis	AMNH 494570	Breast	Purple	Male
Porphyrolaema porphyrolaema	AMNH 43769	Throat	Purple	Male
Lipaugus streptophorus	AMNH 156366, ANSP 162611	Undertail coverts	Rosy pink	Male

transferred to 70 % ethanol (e.g. *Haematoderus militaris* and *Phoenicircus carnifex*).

Pigment extraction

Feather carotenoids were extracted and analyzed using the protocols described previously by LaFountain et al. (2010). Briefly, feathers were washed by soaking in 100 mL of technical grade ethanol for ~15 min and then patted dry. This process was then repeated using technical grade hexanes. After washing, the colored portions were removed with scissors, placed into 15 mL glass test tubes, and covered with acidified pyridine. The tubes were then placed into a 90 °C water bath until loss of pigmentation from the feathers appeared complete which took anywhere from 30 min to 3 h. After 3 h, it was assumed that any remaining pigmentation was not due to carotenoids. The acidified pyridine containing the carotenoid pigments was then added to a mixture of HPLC grade methyl *tert*-butyl ether (MTBE, Fisher Scientific) and water (3:1 v/v) for the purpose of removing both the acidified pyridine and relatively polar lipids (McGraw and Nogare 2005). The water layer was removed with a pipet, and the organic solvent layer was washed with an equal amount of water two additional times. The MTBE phase was then dried under nitrogen gas and re-dissolved in 14 % acetone in hexanes (v/v) for analysis using the previously described HPLC protocol (LaFountain et al. 2010).

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was conducted on a Waters 600E system equipped with a Waters 2996 photodiode array detector. Initial HPLC analysis of feather extracts was carried out using a Phenomenex Luna normal phase (NP) silica column (5 µm, 250 × 4.6 mm). The mobile phase consisted of a linear gradient increasing from 10 % acetone in hexanes to 20 %

acetone in hexanes over 40 min, with a flow rate of 1.5 mL/min. For preparative HPLC, a Waters Sunfire OBD silica prep column (5 μm, 19 × 100 mm) was used with the same gradient, but the flow rate was increased to 7 mL/min. The injection solvent in both cases was 14 % acetone in hexanes (v/v). Unless otherwise stated, the chromatograms were recorded using a detection wavelength of 450 nm.

Prior to mass spectrometry described below, individual pigments were re-purified on the same HPLC system using a reversed phase (RP) protocol. This separated co-eluting peaks and removed additional lipids. The RP protocol employed a Waters NovaPak C18 column (4 μm, 300 × 3.9 mm) and an isocratic delivery of 99 % solvent A, 1 % solvent B for 15 min followed by a linear gradient to 60 % solvent A, 40 % solvent B over the next 25 min. Solvent A consisted of 87:10:3 (v/v/v) acetonitrile/methanol/water (Fisher Scientific), while solvent B consisted of 100 % ethyl acetate (Fisher Scientific).

Pigment identification

Pigment identifications were based on a comparison of absorption spectra and HPLC retention times with bona fide standards as described in previous work (LaFountain et al. 2010). Mass spectrometry and chemical analysis by saponification (Eugster 1995) and sodium borohydride reduction (Eugster 1995) were used to provide additional support for identifications. Mass spectrometry was conducted on either a Fisons Quattro II mass spectrometer (MassLynx software) or an Applied Biosystems QTRAP 4000 (AB analyst software). Both instruments were outfitted with an atmospheric pressure chemical ionization (APCI) probe. Samples were introduced by direct injection into the Quattro II instrument with the following parameters: cone voltage, 15 V; probe temperature, 480 °C, and source temperature, 110 °C. Samples were injected using an infusion pump at a flow rate of 5–10 μL into the QTRAP 400 with the following parameters: probe temperature, 350 °C; curtain gas, 10 psi; collision gas, low; nebulizer current, 2.0 μA; ion source gas, 30.0 psi; declustering potential, 60 V; and collision energy, 10 eV. Mass spectrometry was used for confirmation of the structure of carotenoids from *Xipholena lamellipennis*, *Cotinga cotinga, Rupicola rupicola, Rupicola peruviana saturata, Rupicola peruviana sanguinolenta, H. militaris, P. carnifex, Pyroderus scutatus scutatus* and *Querula purpurata.*

High-resolution mass spectrometry of the major pigment from *C. cotinga* was conducted on a QT of Premier (Micromass UK, Manchester, UK) in APCI mode. The spectra were analyzed with MassLynx software, V4.1 (Micromass UK, Manchester, UK). The source parameters were: corona current, 30 μA; probe temperature, 500 °C, source block temperature, 110 °C; cone voltage, 35 V; cone gas (N_2), 50 L/h; desolvation gas (N_2), 400 L/h; and collision energy, 8 eV with Argon gas.

^{1}H-NMR spectroscopy was carried out using a Bruker Avance 500 MHz spectrometer on the *R. p. sanguinolenta* pigment which eluted from the HPLC at 16 min. For NMR analysis, the sample was dissolved in acetone-D_6 (Cambridge Isotope Laboratories Inc., USA). TMS was used as an internal standard. Due to the low concentration of the sample, 1,024 scans were averaged to improve the signal-to-noise ratio.

Modeling of metabolic pathways

Metabolic pathways for the physiological alteration of common dietary carotenoids were predicted based on previous literature for birds (Prager et al. 2009; Stradi et al. 1995, 1996, 2001; Andersson et al. 2007; Hudon et al. 2007). We then estimated the equilibrium constants of the reactions in each of the hypothesized metabolic pathways assuming the relative frequencies found in plumage represented stable equilibrium concentrations. The equilibrium constant (K) of a metabolic reaction A $\leftrightarrow$ B is an intrinsic parameter of the reaction, and is equal to the ratio of the amount of the product (B) to the amount of the reactant (A): $K = B/A$, where A and B can be expressed in terms of mass, concentration, or relative percentages.

The 19 total reactions in the four proposed carotenoid metabolic pathways consist of six or seven different classes of reactions: (1) C4 (C4′)-ketolation; (2) C3 (C3′)-O-methylation; (3) C2, C3 (C2′, C3′)-didehydrogenation; (4) β-hydroxyl dehydrogenation; (5) ε-hydroxyl dehydrogenation; (6) hydroxylation; and (7) C6-dehydration and re-arrangement to a retro configuration.

The equilibrium constants of these reactions are denoted K_1 through K_7, respectively. At certain stages in some pathways, the same reaction can occur at either of two sites on the same precursor molecule, e.g., once to either β-ring in the C4 (C4′)-oxygenation of β-carotene or zeaxanthin, the C3 (C3′)-O-methylation of astaxanthin, or the dehydrogenation of pompadourin. Consequently, these reactions have an equilibrium constant that will be twice that for the same class of reaction with a single reaction site later in that pathway, or in a different pathway.

For each pathway, we calculated the relative amount of each metabolite at equilibrium as a function of the different equilibrium constants (see Supplementary Table 1). Using the presence or absence of the carotenoids from all pathways in the plumage of each species, some inferences can be made about the metabolic mechanisms and equilibrium

constants for each species. If the dietary precursor and all products of any entire pathway are missing from a plumage, then we concluded that this species does not concentrate, mobilize, or utilize these precursor molecules in the pigmentation of its plumage. If a specific molecule within a given pathway is not detected, we can conclude one of the following:

1. If the precursor molecule is detected, and if all other molecules downstream cannot be detected, then the reaction which produces the downstream metabolites has a very small equilibrium constant (virtually zero). For example, in yellow *Pipreola aureopectus* unmodified lutein and zeaxanthin are deposited directly into the feathers with no detectable metabolic products. Therefore, we hypothesize that the equilibrium constants of the C4-O-methylation and hydroxyl dehydrogenation reactions, K_1 and K_4, respectively, of lutein and zeaxanthin are zero. A K value of zero is equivalent to the hypothesis that the appropriate enzyme either does not exist in this species, or is not expressed in the appropriate place and time.
2. If one of the products downstream of a missing precursor molecule is detected, then the downstream reaction has a very large equilibrium constant compared to the preceding equilibrium constants. For example, in *X. punicea*, we cannot detect any lutein but we can detect xipholenin, which is derived from lutein. Therefore, we can assume that lutein was available for modification and estimate that the equilibrium constants for the reactions in the pathway to the formation of xipholenin (K_1 and K_2) are very large.
3. If neither the precursor nor any of the related downstream products can be detected, we cannot conclude anything about the equilibrium constant of the upstream or the downstream reaction. For example, in *X. punicea*, we cannot conclude anything about the equilibrium constants for the hydroxylation (K_6) or the re-arrangement to retro-constitution reactions (K_7).

Assuming that the enzymatic reactions achieve similar equilibria within the different pathways from different precursor molecules, we can estimate multiple values of a single K_n from different pathways in some species. For example, in *X. punicea*, we can estimate the equilibrium constant for the dehydrogenation reaction (K_3) by calculating the ratios of three different products and their precursors: (1) 2,3-didehydro-xipholenin to xipholenin; (2) cotingin to 2,3-didehydro-pompadourin; (3) 2,3-didehydro-pompadourin to pompadourin. The congruence among these values provides an independent verification of the method.

In some cases, the estimated equilibrium constants were inconsistent with other aspects of the data, i.e., the estimated equilibrium constants predicted the existence of additional molecules that were not observed. In most cases, the predicted amounts were very small, and probably close to the detection limit of the methods. For example, based on the estimated K values in *X. punicea*, we should expect to detect adonixanthin in its plumage at the amount of $6.1/K_1$ percent. However, K_1 is estimated to be very large, which means that the amount of adonixanthin would be very small and below the detection limit. In a few other instances, the predicted amounts are large, and incongruent with the observed data. For example, in *R. peruviana,* the estimated values of K_1 and K_2 predict the presence of substantial amounts of double methoxylated derivatives of zeaxanthin within the plumage. In these cases, the complete absence of these expected molecules in the pathway imply that some other feature of the reactions have changed or have been regulated, either through evolution of differential selectivity of the enzymes (preventing dual reactions), or sequestering carotenoid molecules in a way that prevent the occurrence of other expected reactions within the pathway.

Reflectance measurement and color space modeling

The reflectance spectra of carotenoid patches from museum study skins, or from groups of isolated feathers from study skins, were measured using an S2000 Ocean Optics spectrometer with an Ocean Optics DH-2000Bal deuterium-halogen light source (Ocean Optics, Dunedin, FL, USA). Reflectance was measured at normal incidence to the plumage using a bifurcated illumination/reflectance optical fiber. The optical fiber was held in an aluminum block that eliminated other illumination. The fiber was approximately 6 mm above the plumage, illuminating a circular patch 3 mm in diameter. Each data point between 300 and 700 nm was recorded to obtain the reflectance spectra for the patch.

We modeled avian perception of color using a tetrahedral color space (Stoddard and Prum 2008, 2011), which provides a quantitative representation of sensory experience. We used the free share-ware computer program TetraColorSpace 1. 0 for MATLAB 7 software (Stoddard and Prum 2008, 2011). (Program is available from the authors). The idealized stimulus, Q_I, of each color cone type was estimated by the reflectance spectrum of a plumage patch:

$$Q_I = \int_{300}^{700} R(\lambda) C_r(\lambda) d\lambda, \quad (1)$$

where $R(\lambda)$ is the reflectance spectrum of the plumage patch, and $C_r(\lambda)$ is the spectral sensitivity function of each cone type r. $R(\lambda)$ and $C_r(\lambda)$ functions were normalized to

have integrals of 1. We assumed a standard, constant illumination across all visible wavelengths. For each plumage color, the idealized stimulation values of the four color cones—Q_I—were normalized to sum to one, yielding relative [*uv/v s m l*] values.

The [*uv/v s m l*] values of each reflectance spectrum were converted to a color point with spherical coordinates θ, ϕ, and r, which define a color vector in the tetrahedral color space. This tetrahedral geometry places the achromatic point of equal cone stimulation—white, black, or gray—at the origin and the *uv/v* vertex along the vertical z axis. Each color has a hue and saturation. Hue is defined as the direction of the color vector, given by the angles θ and ϕ, which are analogous to the longitude and latitude, respectively. Saturation, or chroma, is given by the magnitude of r, or its distance from the achromatic origin. Because the color space is a tetrahedron and not a sphere, different hues vary in their potential maximum chroma, or r_{max} (Stoddard and Prum 2008, 2011).

We estimated the avian-perceived diversity of cotinga carotenoid plumage coloration and separate classes of carotenoid coloration (e.g., all non-ketocarotenoid xanthophyll colors) by calculating the volume of color space occupied by the minimum convex polygon containing all relevant color points (Stoddard and Prum 2008, 2011). We also report the contribution of each class of pigments as a proportion of the total color space occupied by all carotenoid color points. We used Robinson projections to view the distribution of hues independent of saturation (Endler et al., 2005; Stoddard and Prum 2008, 2011) The Robinson projection is a compromise between equal-area and conformal projections of the surface of a sphere in 2D, and is a useful tool for visualizing hue variation in color data. Following Ödeen and Håstad's (2003) sequence of the SWS1 opsin of *R. rupicola,* the cotingas were assumed to have the standard, violet-cone type color visual system.

Phylogenetic evolution of metabolic pathways

The diversity of cotinga carotenoid plumage precursor composition and metabolic modifications are analyzed phylogenetically using a phylogeny of Tello et al. (2009), which is highly congruent with previous hypotheses (Prum et al. 2000, Ohlson et al. 2007). Some slight modifications were required to accommodate missing taxa. The phylogenetic hypothesis from Tello et al. (2009) was input into MacClade 4.08a, along with a matrix for the presence and absence of precursor carotenoids in their plumage and requirement of each hypothesized metabolic transformations to produce that species (K_1 through $K_7 > 0$). Evolutionary hypotheses for origin of character diversity were calculated using parsimony in MacClade.

Results

Carotenoid diversity

A total of 16 different xanthophyll carotenoids were isolated and identified from the feathers of 25 species of cotingas (Fig. 2; Table 2). These 16 molecules include two common dietary xanthophylls (lutein and zeaxanthin), four well-known and broadly distributed avian ketocarotenoids (canthaxanthin, astaxanthin, α-doradexanthin, and adonixanthin), canary xanthophylls A and B, rhodoxanthin, and seven 4-methoxy-ketocarotenoids including a new, previously undescribed molecule isolated from the Andean Cock-of-the-Rock *R. peruviana.*

The details of the identification of the pigments found in each species examined are presented in the Electronic Supplementary Material, but the details of the new molecule from *R. peruviana* are highlighted here. The HPLC peak collected at 14 min in *R. p. saturata* (Figure S18) and 16 min in *R. p. sanguinolenta* (Figure S19) had an HPLC retention time, absorption spectrum, and mass (596 *m/z*) consistent with that of the pigment xipholenin (3′-hydroxy-3-methoxy-β,ε-carotene-4-one). After chemical reduction in methanol using sodium borohydride, the sample showed a shift in the absorbance spectrum of ~12 nm to shorter wavelength, which is consistent with the presence of one conjugated carbonyl. However, the sodium borohydride reduction revealed a β-carotene chromophore and not an α-carotene chromophore as would be expected for xipholenin. Consequently, this pigment is more likely 3′-hydroxy-3-methoxy-β,β-carotene-4-one, which is a novel molecule. The presence of a methoxyl group on the pigment collected from *R. p. sanguinolenta* was confirmed by ^{1}H-NMR. Because 3′-hydroxy-3-methoxy-β,β-carotene-4-one is a new pigment that has been described from the cotinga genus *Rupicola,* we propose here to call this molecule rupicolin (Fig. 2)

Carotenoid distribution in cotingas

The number of carotenoids found in a single plumage color patch of any single species varied from one—either lutein or cotingin—to eight different ketocarotenoids in *X. punicea* (Table 2). Lutein was found alone in yellow plumages of six different genera (Table 2). Lutein was found in combination with zeaxanthin in yellow feathers of the fruiteaters *P. aureopectus* and *P. formosa* (Figures S7–S9) and also in combination with a pigment tentatively identified as α-doradexanthin in orange plumage of *Pipreola whitelyi* (Figure S12). An additional, third carotenoid from *P. whitelyi* could not be identified with the limited material available. The red plumage patches of *P. formosa* and *P. chlorolepidota* contained a pigment in addition to lutein

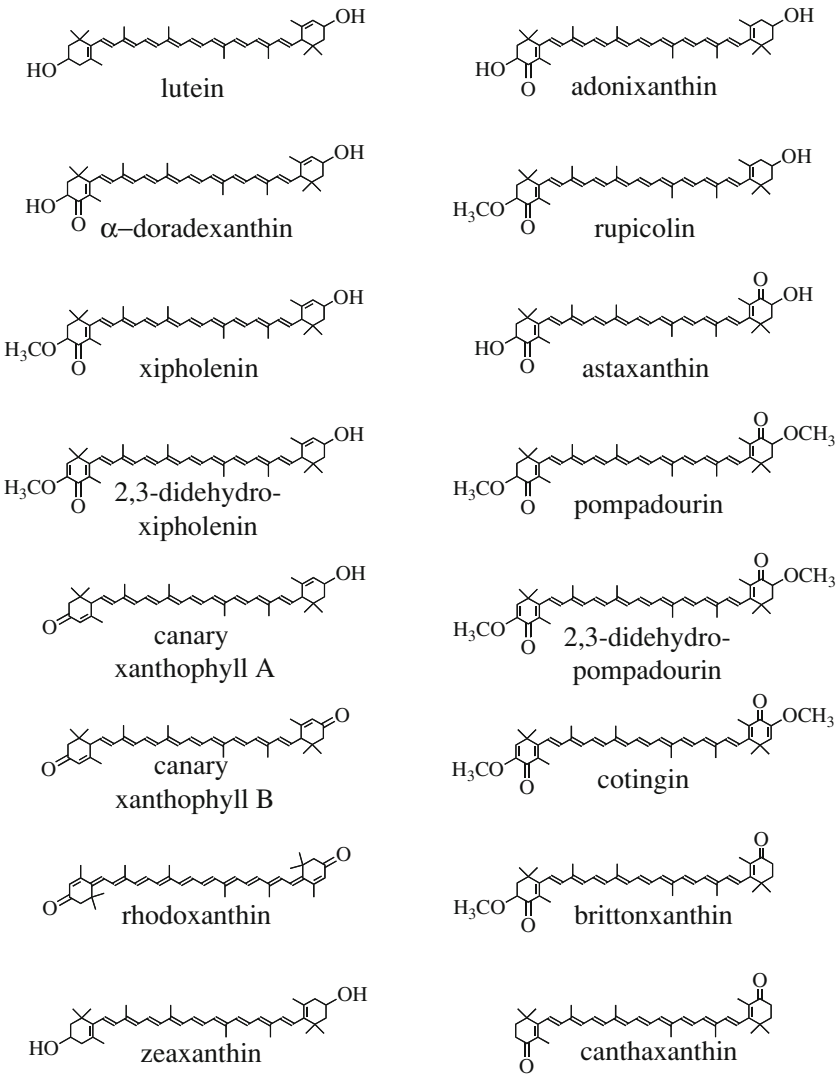


Fig. 2 Molecular structures of the sixteen carotenoid pigments isolated or detected by HPLC from cotinga plumages (Cotingidae)

and zeaxanthin; this pigment was not α-doradexanthin, but it could not be identified without additional material. Yellow plumage of female Three-wattled Bellbird *Procnias tricarunculata* was characterized by the presence of canary xanthophylls A and B (Figure S13). The orange throat plumage of the Red-ruffed Fruitcrow *P. scutatus* contained lutein, canthaxanthin, canary xanthophyll A, and α-doradexanthin; the two subspecies of *P. scutatus* examined varied in relative frequencies of each pigment (Figures S14 and S15).

The brilliantly red plumage of male Guiana Red Cotinga (*P. carnifex*) contained rhodoxanthin and very small amounts of lutein (Figure S16). The retro configuration of rhodoxanthin, which is rare in animals, creates a conjugated chain of 12 alternating carbon–carbon double bonds, creating a red-shift in its absorbance and a deeply red visible color. The brilliant orange plumage of male Guianan Cock-of-the-Rock (*R. rupicola*) contained five carotenoids (Figure S17)—lutein, canthaxanthin, canary xanthophyll A, α-doradexanthin, and xipholenin—which is the most abundant methoxy-ketocarotenoid in *X. punicea.* However, its sister species, *R. peruviana,* has a deep orange-red plumage, which is even darker in the populations of the western slopes of the Andes (*R. p. sanguinolenta*) than in the populations of the eastern slopes of Andes (e.g., *R. p. saturata*). The red plumage of both subspecies of *R. peruviana* contained a mixture of four carotenoids that were entirely different from those found in *R. rupicola* plumage—zeaxanthin, adonixanthin, xipholenin, and the newly described methoxy-ketocarotenoid, rupicolin (see above, Figs. 2, S18 and S19).

The deeply purple and black plumages of two other species of *Xipholena*—the White winged (*X. atropurpurea*) and the White-tailed (*X. lamellipennis*) Cotingas, respectively—were similar in carotenoid content to *X. punicea* previously described in detail by LaFountain et al. (2010), except that they lacked astaxanthin, 2,3-didehydro-xipholenin, and cotingin (Figures S21 and S22). These two

Table 2 Mass percentages of carotenoids by species

Pigments[a]	*Carpornis cucullatus*	*Snowornis subalaris*	*Phibalura flavirostris*	*Ampelion rufaxilla*	*Ampeliodes tschudii*	*Tijuca atra*	*Pipreola aureopectus*	*Pipreola formosa* (yellow belly)	*Pipreola formosa* (orange throat)	*Pipreola chlorolepidota*	*Pipreola whitelyi*	*Procnias tricarunculata*	*Pyroderus scutatus scutatus*	*Pyroderus scutatus occidentalis*
1	100	100	100	100	100	100	90	89	79	72	25		11	10
2											48		51	22
3														
4														
5												26	21	9.5
6												74		
7														
8							10	11	8	19				
9														
10														
11														
12														
13														
14														
15														
16													6.6	52
17									13	9	27		10	6.4

Pigments[a]	*Phoenicircus carnifex*	*Rupicola rupicola*	*Rupicola peruviana saturata*	*Rupicola peruviana sanguinolenta*	*Xipholena punicea*	*Xipholena lamellipennis*	*Xipholena atropurpurea*	*Haematoderus militaris*	*Querula purpurata*	*Cotinga cotinga*	*Cotinga maculata*	*Cotinga amabilis*	*Porphyrolaema porphyrolaema*	*Lipaugus streptophorus*
1	4.4	16												
2		32												
3		17			28	18		25	37					
4					18		23		14					
5		21												
6														
7	96							9						
8			26	11										
9			37	37										
10			28	28										
11					6.1									
12					11	12	13	39	26					
13					13	4.1	4.9	9.5					8	23
14					7.4					100	100	100	92	77
15					8.2	5.2	6.5	14	14					
16		11			7.4	21	30							
17		3.3	8.7	24		40	23	3	9.5					

[a] Pigments are designated as follows: (1) lutein, (2) α-doradexanthin, (3) xipholenin, (4) 2,3-didehydro-xipholenin, (5) canary xanthophyll A, (6) canary xanthophyll B, (7) rhodoxanthin, (8) zeaxanthin, (9); adonixanthin, (10) rupicolin, (11) astaxanthin, (12) pompadourin, (13) 2,3-didehydro-pompadourin, (14) cotingin, (15) brittonxanthin, (16) canthaxanthin, and (17) unidentified

species are distinct in having very dark or black plumage which is a combination of intense carotenoid deposition and perhaps melanins (Völker 1952, 1955; Schmidt 1956).

The crimson-magenta plumages of the Purple-throated Fruitcrow (*Q. purpurata*) and the Crimson Fruitcrow (*H. militaris*) each contained four of the six methoxy-carotenoids previously described from *X. punicea*: *H. militaris* contained brittonxanthin, pompadourin, xipholenin, and 2,3-didehydro-pompadourin (Figure S23); *Q. purpurata* was found to contain brittonxanthin, pompadourin, xipholenin, and 2,3-didehydro-xipholenin (Figure S24). Additionally, *H. militaris* was found to contain a small amount of rhodoxanthin (Table 2), which was identified based on comparison of the absorption spectrum and HPLC retention time with those of a bona fide standard. No precursors of rhodoxanthin were observed.

The deep purple throat or belly plumages of the Purple-breasted Cotinga (*C. cotinga*), the Lovely Cotinga (*C. amabilis*), Banded Cotinga (*C. maculata*), contained only cotingin (Figures S25–S27). The deep purple throat patch of male Purple-throated Cotinga (*Porphyrolaema porphyrolaema*) contained mostly cotingin with some 2,3-didehydro-pompadourin (Figure S28). Surprisingly, the rosy pink collar and undertail coverts of the Rose-collard Piha (*Lipaugus streptophorus*) contained similar quantities of cotingin and 2,3-didehydro-pompadourin (Figure S29), despite their entirely different appearance from *Cotinga* and *Porphyrolaema*, strongly implying a role for binding of these carotenoids by feathers proteins in determining methoxy-carotenoid plumage coloration.

Proposed metabolic pathways

All cotinga feather carotenoids are hypothesized to be derived from four different dietary precursors: lutein, zeaxanthin, β-carotene, and β-cryptoxanthin (Figs. 3, 4). In total, the hypothesized metabolic pathways require six independent enzymatic reactions (labeled in colors in Figs. 3, 4): (1) C4 (C4′)-oxygenation (green), (2) C3 (C3′)-O-methylation (red), (3) C2,C3 (C2′, C3′)-didehydrogenation (blue), (4) β-hydroxyl dehydrogenation (orange), (5) ε-hydroxyl dehydrogenation (yellow), (6) hydroxylation (purple), and (7) C6-dehydration and re-arrangement to a retro configuration (brown).

Several alternative metabolic pathways were ruled out as unlikely because of the absence of any trace of the predicted molecules. For example, the absence of any

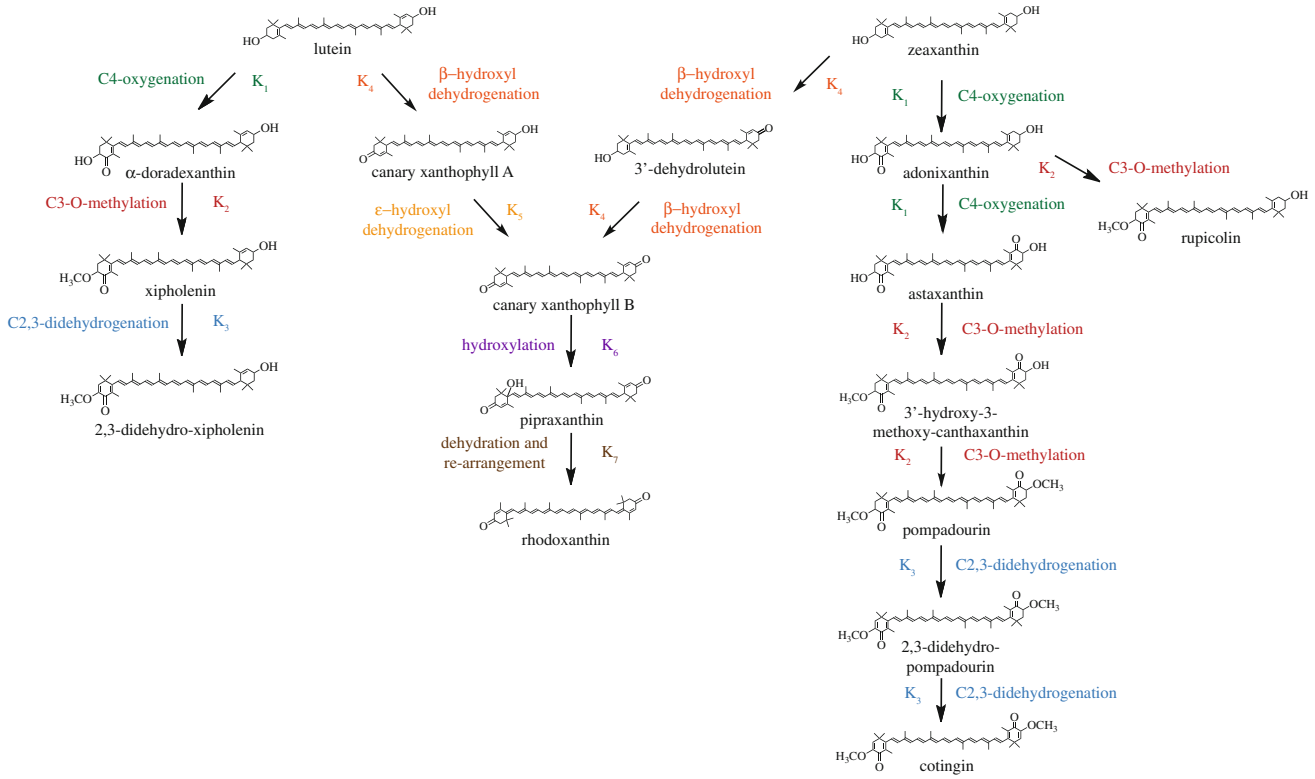


Fig. 3 The proposed physiological pathways for the metabolic transformation of the dietary precursors lutein and zeaxanthin to produce the carotenoid molecules found in the plumages of various species of cotingas (based on Stradi et al. 1995, 1996, 2001; Andersson et al. 2007; Hudon et al. 2007). Pathways require six metabolic reactions: (1) C4-oxygenation (*green*), (2) C3-O-methylation (*red*), (3) C2,C3-didehydrogenation (*blue*), (4) β-hydroxyl dehydrogenation (*orange*), (5) ε-hydroxyl dehydrogenation (*yellow*), (6) C6-hydroxylation (*purple*), and (7) dehydration followed by retro conversion (*brown*)

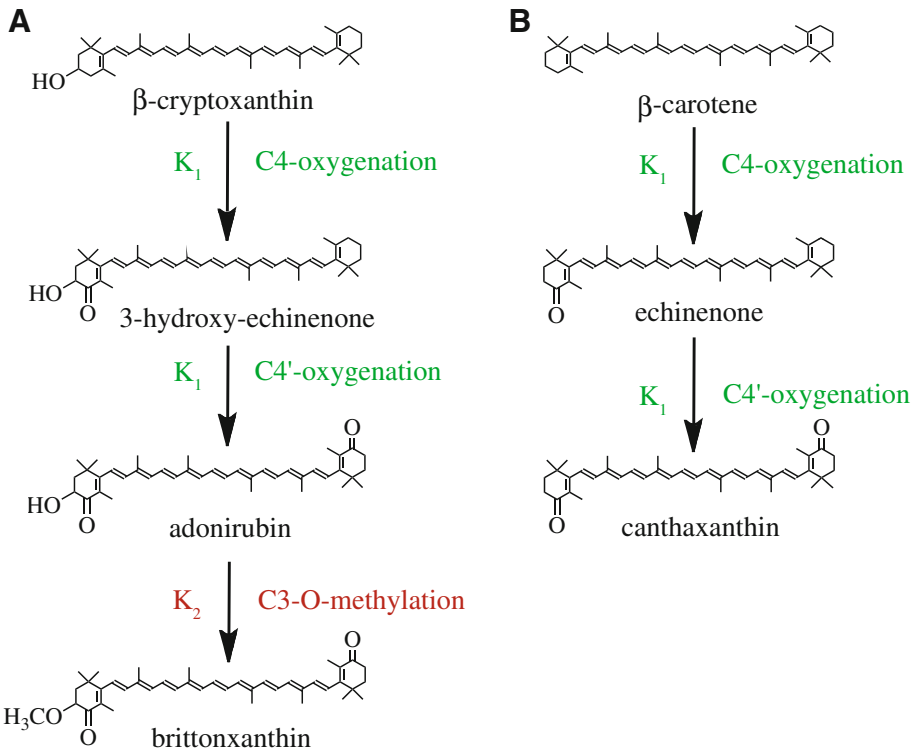


Fig. 4 The proposed physiological pathways for the metabolic transformation of the dietary precursors, **a** β-cryptoxanthin and **b** β-carotene to produce the carotenoid molecules found in the plumages of various species of cotingas (based on Stradi et al. 1996, 2001; Andersson et al. 2007; Prager et al. 2009). These pathways require three metabolic reactions: (1) C4-oxygenation (*green*), (2) C3-O-methylation (*red*)

methoxy-carotenoids lacking a C4-carbonyl (such as methoxy-zeaxanthin detected in the human macula) indicates the enzyme that performs C3-O-methylation can only proceed using 3-hydroxy-4-keto-β-rings.

There are two lutein pathways (Fig. 3). One produces xipholenin, and the other produces canary xanthophylls and rhodoxanthin. In the lutein-xipholenin pathway, lutein first undergoes C4-oxygenation to produce α-doradexanthin. Subsequent C3-O-methylation produces xipholenin, and then C2–C3-didehydrogenation produces 2,3-didehydro-xipholenin. In the lutein-rhodoxanthin pathway (Fig. 3), β-hydroxyl is dehydrogenated to produce canary xanthophyll A, followed by C3′-ε-hydroxyl dehydrogenation to produce canary xanthophyll B. Following the hypothesis of Hudon et al. (2007), canary xanthophyll B is then transformed by hydroxylation at C6 to produce 6-hydroxy-ε,ε-carotene-3,3′-dione, or pipraxanthine, which is subsequently dehydrogenated at C6 and rearranged into a retro configuration to produce rhodoxanthin.

There are two alternative zeaxanthin pathways: one which yields four distinct methoxy-carotenoids found in cotinga plumages and an alternative pathway to canary xanthophyll B and rhodoxanthin (Fig. 3). In the former, C4-oxygenation of zeaxanthin produces adonixanthin and astaxanthin, successively. Astaxanthin is then modified by C3-O-methylation to produce 3′-hydroxy-3-methoxy-canthaxanthin (which has yet to be found in any bird plumage), and C3′-O-methylation to produce pompadourin. Pompadourin can be transformed by C2–C3-didehydrogenation of the two β-rings to produce 2,3-didehydro-pompadourin and cotingin, successively. Alternatively, adonixanthin can be modified by C3-O-methylation to produce rupicolin. In the alternative rhodoxanthin pathway, zeaxanthin is converted by successive β-hydroxyl dehydrogenation of C3 and C3′ to produce 3′-dehydrolutein and canary xanthophyll B. Subsequently, pipraxanthine and rhodoxanthin are produced by the same mechanisms as above (Fig. 3).

In the β-cryptoxanthin to brittonxanthin pathway (Fig. 4a), β-cryptoxanthin is modified by C4 and C4′-oxygenation to make 3-hydroxy-echinenone and adonirubin successively. Adonirubin is modified by C3-O-methylation to produce brittonxanthin. Lastly, canthaxanthin is produced by C4 and C4′-oxygenation of β-carotene via the intermediate echinenone (Fig. 4b).

Quantitative modeling of carotenoid metabolism

The variation in plumage coloration among species is hypothesized to be a consequence of the evolution of the physiological mechanisms of concentration, transport, modification and deposition of carotenoids. By assuming that the relative frequencies of different carotenoids in the feathers were the result of a metabolic or physiological equilibrium, we created a quantified description of the variation in carotenoid composition of each species using: (1) the relative composition of precursor and downstream carotenoids from each of the four pathways, and (2) the

Table 3 Percent precursor composition of total feather carotenoids for cotinga species studied, and estimated equilibrium constants for the metabolic transformation reactions (K_1 through K_7) to produce the observed carotenoid frequencies (see Table 2)

Species	Percent precursor composition					Estimated equilibrium constants						
	Lutein	Zeaxanthin	β-cryptoxanthin	β-carotene	Unknown	K_1	K_2	K_3	K_4	K_5	K_6	K_7
Carpornis cucullatus	100					0	Ø	Ø	0	Ø	Ø	Ø
Snowornis subalaris	100					0	Ø	Ø	0	Ø	Ø	Ø
Phibalura flavirostris	100					0	Ø	Ø	0	Ø	Ø	Ø
Ampelion rufaxilla	100					0	Ø	Ø	0	Ø	Ø	Ø
Ampelioides tschudii	100					0	Ø	Ø	0	Ø	Ø	Ø
Tijuca atra	100					0	Ø	Ø	0	Ø	Ø	Ø
Pipreola aureopectus	89.8	10.2				0	Ø	Ø	0	Ø	Ø	Ø
Pipreola formosa (belly)	88.7	11.3				1.0	Ø	Ø	0	Ø	Ø	Ø
Pipreola formosa (throat)	78.7	7.7			13.6	2.0	Ø	Ø	0	Ø	Ø	Ø
Pipreola chlorolepidota	71.8	19.4			8.7	3.0	Ø	Ø	0	Ø	Ø	Ø
Pipreola whitelyi	73.3				26.7	1.9	0	Ø	0	Ø	Ø	Ø
Procnias tricarunculata	100 (25)	(75)				0	Ø	Ø	Large	2.9 (0)	0	Ø
Pyroderus s. scutatus	83.1		6.6		10.3	4.6	0	Ø	0.9	0	Ø	Ø
Pyroderus s. occidentalis	41.6		52		6.4	2.1	0	Ø	0.5	0	Ø	Ø
Phoenicircus carnifex	100					0	Ø	Ø	<Large	Large	Large	Large
Rupicola rupicola	85.4		11.3		3.3	2.0	0.5	0	0.7	0	Ø	Ø
Rupicola peruviana saturata		100				0.7	0.8	Ø	0	Ø	Ø	Ø
Rupicola p. sanguinolenta		100				0.7	0.3	Ø	0	Ø	Ø	Ø
Xipholena punicea	46.4	37.6	7.4	8.2		Large	1.0	0.6	0	Ø	Ø	Ø
Xipholena lamellipennis	17.8	15.7	21.4	5.2	39.8	Large	Large	0.4	0	Ø	Ø	Ø
Xipholena atropurpurea	22.6	17.7	30	6.5	23.1	Large	Large	0.4	0	Ø	Ø	Ø
Haematoderus militaris	34 (25)	48.6 (54)	14.3		3.1	Large	Large	0.1	Large	(Large)	Large	Large
Querula purpurata	50.8	25.9	13.8		9.5	Large	Large	0.4	0	Ø	Ø	Ø
Cotinga cotinga		100				Large	Large	Large	0	Ø	Ø	Ø
Cotinga maculata		100				Large	Large	Large	0	Ø	Ø	Ø
Cotinga amabilis		100				Large	Large	Large	0	Ø	Ø	Ø
Porphyrolaema porphyrolaema		100				Large	Large	11.6	0	Ø	Ø	Ø
Lipaugus streptophorus		100				Large	Large	3.3	0	Ø	Ø	Ø

Ø indicates that K_n values is inestimable because of the lack of upstream precursors. Alternative dietary compositions and K values are given in parentheses for *Procnias tricarunculata* and *Haematoderus militaris*

estimated magnitudes of the equilibrium constants of the six enzymatically catalyzed metabolic modifications within the four pathways. The results are estimates of the relative contributions of the four precursors to total plumage carotenoid composition, and estimate of equilibrium constants K_1 through K_7 for each species (Table 3; colored labels in Figs. 3, 4).

A large number of species from six genera have yellow plumages created by depositing 100 % unmodified dietary lutein. Yellow plumages of *Pipreola* deposit lutein with an additional 7–20 % unmodified zeaxanthin in their yellow plumages. For all these species, the metabolic reaction constants K_1 through K_7 are 0 or inestimable, but there is some evolutionary variation in the capacity to concentrate

and selectively deposit lutein and zeaxanthin. Within *Pipreola*, *P. whitelyi* has evolved to produce its the orange-red plumage with a substantial value of K_1 (0.45), or C4-oxygenation, for the production of α-doradexanthin from lutein. *P. whitelyi* does not use zeaxanthin.

For those species in which canary xanthophyll B or rhodoxanthin are present, additional analysis is required to differentiate the likelihood of the two alternative pathways derived from either lutein or zeaxanthin. The canary xanthophyll A and B plumage of female *P. tricarunculata* could be produced exclusively from lutein precursor molecules with a very large K_4 value (supported by the absence of lutein) and a K_5 value of 2.9. All other reactions are zero or inestimable. Alternatively, it also possible for *Procnias* to produce canary xanthophyll A from lutein and canary xanthophyll B from zeaxanthin with a very large value of K_4 and differential concentration of dietary lutein and zeaxanthin. Dietary deprivation experiments would be required to test these two alternatives.

Rhodoxanthin in the plumage of *P. carnifex* could be derived from either lutein or zeaxanthin (Fig. 3). The low but measurable quantity of lutein (<5 %) in *Phoenicircus* plumage would be anomalous if rhodoxanthin were derived from zeaxanthin. If the rhodoxanthin production from zeaxanthin were efficient enough to produce 96 % rhodoxanthin in the plumage, then we would also expect to see production of canary xanthophyll A by β-hydroxyl dehydrogenation of any lutein that was also sequestered during the production of plumage carotenoids. Instead we see only a small fraction of lutein. However, this lutein anomaly can be appropriately explained if rhodoxanthin is derived from lutein. The extremely high concentration of rhodoxanthin (96 %) indicates that large values of K_5, K_6, and K_7, but a somewhat smaller value of K_4 (to account for the residue of lutein), and a K_1 value of zero. So, the existence of trace lutein in *Phoenicircus* provides some logical support for the derivation of rhodoxanthin from this precursor.

The plumage carotenoid compositions of the remaining cotinga species require highly variable and sometimes novel combinations of precursor concentrations and kinetic constants. *R. rupicola* is produced by a majority of lutein and $\sim$11 % β-cryptoxanthin precursors with large values for K_1 (2.0), moderate values for K_2 (0.5) and K_4 (0.7), and zero values for K_3, K_5, K_6, and K_7. These values predict the presence of a trace amount of echinenone, which was not detected, but the predicted levels are within the experimental error.

In contrast, the plumages of both *R. peruviana* subspecies are based entirely on zeaxanthin precursor molecules (not used in *R. rupicola*) with a moderate values for K_1 (0.7 in both subspecies) and K_2 (0.7 in *saturata*; 0.3 in *sanguinolenta*). These K_1 and K_2 values are required to explain the presence of >25 % of unmodified zeaxanthin in the plumage. However, reaction constants of this magnitude also predict the presence of similar frequencies of astaxanthin, 3′-hydroxy-3-methoxy-canthaxanthin, and pompadourin, which were not detected in *R. peruviana* plumages (although the percent of unidentified material in *R. p. sanguinolenta* is higher than other species). This incongruence implies that some additional metabolic process is involved to prevent these reactions, such as pigment sequestration or the evolution of additional enzyme specificity.

The carotenoid composition of *P. scutatus* is based on lutein and β-cryptoxanthin, but the two subspecies analyzed differ strikingly in contribution of β-cryptoxanthin: *P. scutatus scutatus* (6 %), and *P. scutatus occidentalis* (52 %). Interestingly, feathers in both species have a substantial phaeomelanin pigmentation. *P. s. occidentalis* also has a much lower total carotenoid content. Despite differences in composition and quantity, the carotenoid frequencies of both subspecies support very similar estimates of the reaction constants—substantial values for K_1 (4.6, 2.1, respectively), moderate values for K_4 (0.9, 0.5;), and zero values for K_2, K_3, K_5, K_6, and K_7.

The remaining cotinga species with carotenoid plumage form three distinct pigment classes with small variations within each class. The feather carotenoids of the three *Cotinga* species, *Porphryolaema,* and *L. streptophorus* are derived entirely from zeaxanthin. The pure cotingin composition in plumages of *Cotinga* is the result of inestimably large values of K_1, K_2, and K_3. The mixtures of cotingin and 2,3-didehydro-pompadourin in *Porphryolaema,* and *L. streptophorus* result from very large values of K_1 and K_2, but smaller, estimable values of K_3—11.6 and 3.3, respectively.

Querula purpurata and *H. militaris* have similar precursor compositions and estimates of K_1, K_2, and K_3. *Querula purpurata* has a precursor composition of 50 % lutein, 26 % zeaxanthin, and 14 % β-cryptoxanthin, and large values of K_1 and K_2, modest value of K_3 (0.4) and a zero value for K_4, preventing the production of rhodoxanthin as found in *Haematoderus. Haematoderus* plumage carotenoids are also derivatives of lutein, zeaxanthin, and β-cryptoxanthin. The precise precursor frequencies are difficult to estimate because of ambiguity over whether the 9 % rhodoxanthin is derived from lutein (via K_4 and K_5) or from zeaxanthin (via K_4 alone). If rhodoxanthin is derived from lutein, then the precursor composition are 34 % lutein, 48.5 % zeaxanthin, and 14.3 % β-cryptoxanthin, whereas if rhodoxanthin is derived from zeaxanthin, then the precursor composition would be 25 % lutein, 54 % zeaxanthin, and 14.3 % β-cryptoxanthin. Regardless of the pathway of derivation of rhodoxanthin (i.e. whether $K_5 > 0$ or not), the relative frequency composition of *Haematoderus* plumage carotenoids is the result of very large

values of K_1, K_2, K_4, K_6, and K_7 with a modest value for K_3 (0.1). Alternatively, it is also possible that the minority fraction of rhodoxanthin in *Haematoderus* is of dietary origin. However, the presence of apparently metabolically derived rhodoxanthin in *Phoenicircus,* manakins, and in fruit doves strongly suggests physiological derivation in this cotinga as well (Hudon et al. 2007).

The three species of *Xipholena* have quite complicated carotenoid compositions produced by having substantial but variable fractions of all four dietary precursor molecules and variable K values. The plumage of *X. punicea* has most pigments derived from lutein (46 %) and zeaxanthin (38 %), whereas the plumage of *X. lamellipennis* and *X. atropurpurea* have the largest proportion of pigments derived from β-cryptoxanthin (30 and 21 %, respectively). *X. lamellipennis* and *X. atropurpurea* have large values of K_1 and K_3, a moderate value of K_3 (0.3), and a zero value for K_4. By contrast, *X. punicea* has a large value for K_1 only, an intermediate value of K_2 (0.6), and a moderate value of K_3 (0.6), and a zero value of K_4.

The diversity of pathways involving three different precursors in *X. punicea* provides three independent estimates of K_3: from the conversion of xipholenin to 2,3-didehydro-xipholenin (0.64); the conversion of pompadourin to 2,3-didehydro-pompadourin (0.57); and the conversion of cotingin from 2,3-didehydro-pompadourin (0.59). These strongly concordant values support validity of the model.

These estimated K values predict the presence of additional molecules that were unidentified in all three *Xipholena* species. For example, the presence of 2,3-didehydro-pompadourin in *X. lamellipennis* and *X. atropurpurea* implies the possible occurrence of cotingin and 2,3-didehydro-xipholenin. These molecules could be present but have gone undetected. Because of the scarcity of material available, *lamellipennis* and *atropurpurea* had substantial fractions of unidentified carotenoid molecules. Likewise, the estimated K values of *X. punicea* predict small quantities of adonixanthin, rupicolin, and 3-hydroxy-3-methoxy-canthaxanthin, which were not detected in the sample. The value of K_1 for *X. punicea* also predicts a larger component of α-doradexanthin, which was also not observed in the samples. This substantial deviation from expectation may be the result of selective variation in K_1 for different precursor carotenoids or selective sequestration of certain molecules.

Reflectance spectra and color space analyses

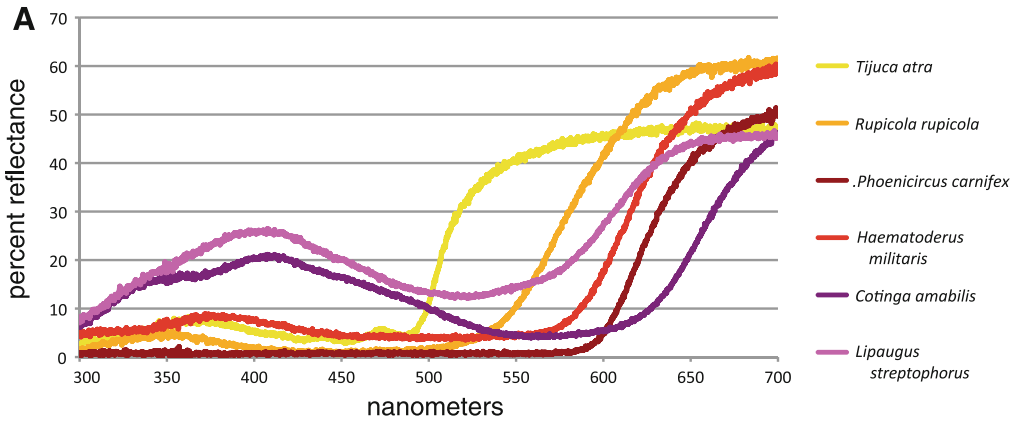
An analysis of the diversity of cotinga carotenoid plumage reflectance using tetrahedral avian color space can provide insights into the consequences of biochemical variation in plumage pigmentation on the color perceptions made by cotingas themselves. The carotenoid plumage patches of cotinga vary extensively in reflectance based on the variation in breadth and position of the short wavelength absorption creating yellow, orange, red, purple, and rosy pink plumage colors (Fig. 5a; Supplementary Figure S30). We analyzed this color diversity using a tetrahedral model of avian color perception (Stoddard and Prum 2008, 2011) with the violet-cone type visual system model, based on the SWS1 opsin gene sequence of *R. rupicola* (Ödeen and Håstad, 2003) (Fig. 5b).

The distribution of cotinga carotenoid plumage color points in the avian color space document that plumages with similar molecular composition cluster together, creating similar visual perceptions (Fig. 5c–e). Based on this clustering in color space, three classes of carotenoid plumage colors were recognized: (1) simple xanthophylls and canary xanthophylls (yellow dots, Fig. 5c–e); (2) complex 4-ketocarotenoid plumages (orange dots) and rhodoxanthin (red dots) with little or no methoxy-ketocarotenoid content (Fig. 5c–e) and (3) methoxy-ketocarotenoid pigments with little or no other carotenoids (purple dots, Fig. 5c–e).

A figure with the entire data set identified to individual species is provided in the Supplementary Figure S30.

The colors of the simple yellow xanthophyll plumages were distributed linearly from near the achromatic center of the tetrahedron to the edge between the long-wavelength (red) and mid-wavelength (green) vertices of the color space (yellow dots, Fig. 5c). This is expected since yellow colors should be distributed in color space between greens and reds. The colors of complex 4-ketocarotenoid plumages were distributed in color space between the xanthophyll color points and the long-wavelength cone vertex

Fig. 5 **a** Reflectance spectra of a sample of carotenoid pigmented plumage patches of cotingas: *Tijuca atra* yellow (lutein only), *Rupicola rupicola* orange (ketocarotenoid mixture), *Phoenicircus carnifex* red (rhodoxanthin), *Haematoderus militaris* crimson (methoxy-ketocarotenoid mixture), *Cotinga amabilis* purple (cotingin only), and *Lipaugus streptophorus* rosy pink (cotingin and 2,3-didehydro-pompadourin). **b** Tetrahedral avian color space (Stoddard and Prum 2008, 2011). Based on the relative stimulation of the four classes of color channels—*v, s, m, l*—each color can be described by a color vector with spherical coordinates *θ, ϕ, r.* The angles θ and ϕ describe the hue, and the length of the color vector r is a measure of chroma, or saturation (i.e. the difference from achromatic *white/black*). **c** Distribution of the reflectance spectra from 29 cotinga carotenoid plumage patches in the tetrahedral color space divided into three molecular classes: simple xanthophylls and canary xanthophylls (*yellow dots*), complex ketocarotenoids (*orange*) with rhodoxanthin (*red*), and methoxycarotenoids (*purple*). An image with each plumage patch identified to species is provided in Supplementary Figure S30. **d** Robinson projection of the hue (θ and ϕ only) of cotinga carotenoid plumage reflectances by carotenoid type. Methoxy-carotenoids (*purple*) create unique "ultrapurple" hues. **f** The distribution of a cotinga carotenoid colors in comparison with the gamut of other avian carotenoid colors (*gray dots and polyhedron*) (from Stoddard and Prum 2011). Methoxy-carotenoids achieve novel visual colors not produced by other birds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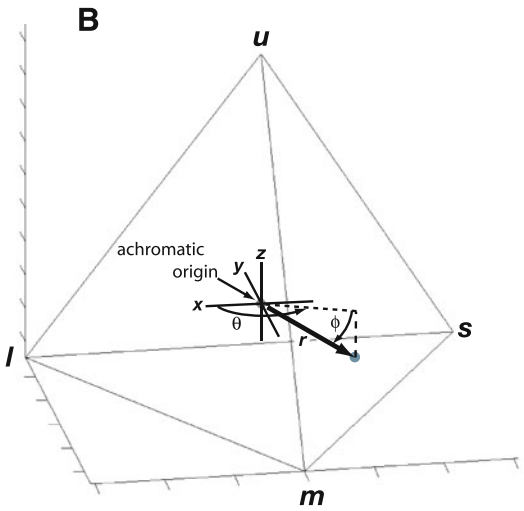
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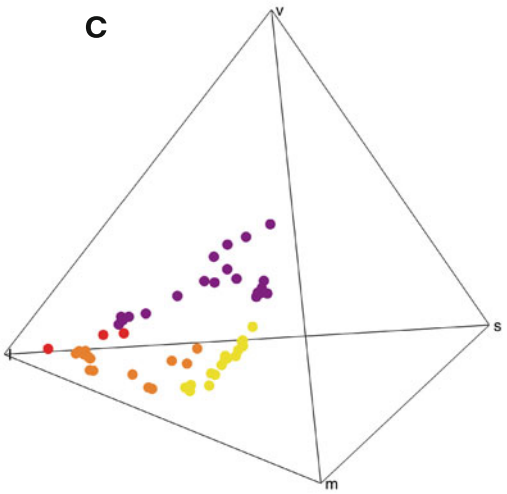
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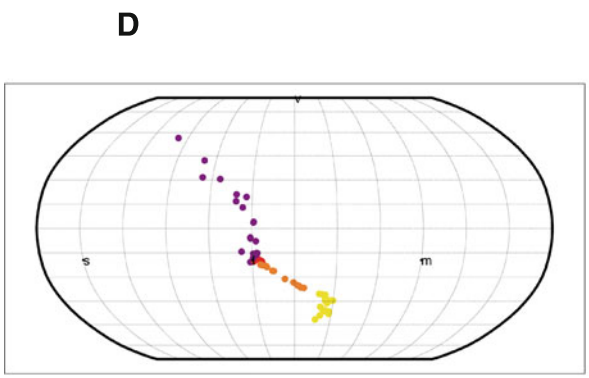
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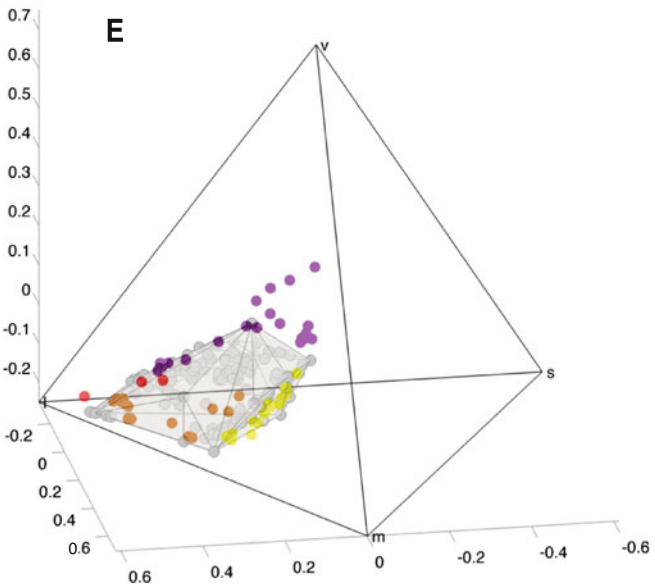
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(orange dots, Fig. 5c). Variations in molecular composition within this class of plumages are registered as an increasing shift toward the long-wavelength vertex. Thus, orange *Pipreola whitelyi* containing modest amounts of α-doradexanthin is closer to the molecularly more similar xanthophyll points, whereas the *Pyroderus* and *Rupicola* plumages that contain a rich diversity of 4-ketocarotenoids (and a small portion of methoxy-carotenoids in *Rupicola*) are much redder in color. Colors of the nearly pure rhodoxanthin plumage of *P. carnifex* are distributed linearly near the long-wavelength vertex (red dots, Fig. 5c); thus, the rhodoxanthin crown of *P. carnifex* is the most extreme saturated red color in the cotinga family.

The colors of plumages composed largely or exclusively of methoxy-carotenoids are distributed in an entirely distinct region of the color space that lies above the ketocarotenoid color points, and extends into the ultraviolet region (above the achromatic origin) and along the "purple" surface of the tetrahedron between the violet, blue, and red vertices (purple dots, Fig. 5c). The plumages of *X. punicea, H. militaris,* and *Q. purpurata*, which have a broader, more complex mixture of different methoxy-carotenoids, are distributed nearer to the long-wavelength vertex, and are more similar in appearance to the rhodoxanthin plumage of *Phoenicircus* (Fig. 5c, red dots). The cotingin-dominated plumages of *Cotinga, Porphyrolaema,* and *L. streptrophorus* have the highest violet and purple components, and are distributed into the upper regions of the color space.

The reflectances of *Xipholena atropurpurea, X. lamellipennis,* and some samples of *C. amabilis* and *P. porphyrolaema* are nearly black, and map in a cluster very closely to the achromatic point at the center of the color space (Fig. 5c, central cluster of purple dots).

The Robinson projection of the color vectors provides a visual map of variation in hue as perceived by birds, i.e., the angular direction of the color vector—independent of saturation—the distance from the achromatic center. The Robinson project of the cotinga carotenoid plumage reflectance spectra demonstrates that molecular differences among the three molecular classes of molecules create substantial differences in avian-perceived hue (Fig. 5d). Beginning with extremely saturated reds, the hues of the methoxy-ketocarotenoid plumages extend in a line in the "purple" and "ultraviolet-purple" region between the blue (s, lower left) and the violet (v, upper) vertices of the color space (Fig. 5d). Thus, the methoxy-carotenoids create a distinct class of "ultrapurple" colors.

The volume circumscribed by the cotinga carotenoid color points provides a quantitative measure of color diversity and the relative contributions of each class of plumages to cotinga color diversity. Following the broad scale phylogenetic pattern in birds and in cotingas specifically (see below), we can hypothesize that the pigment utilization evolves through a series of stages from: (1) simple xanthophyll utilization, (2) diverse ketocarotenoid synthesis and utilization, to (3) methoxy-ketocarotenoid synthesis and utilization.

Accordingly, simple xanthophyll colors occupy only ~1 % of the total cotinga carotenoid color diversity. With the evolutionary addition of diverse ketocarotenoids (including rhodoxanthin), cotinga carotenoid plumage diversity grew to occupy 32 % of its current total. Thus, with the evolution and diversification of methoxy-ketocarotenoids, cotingas effectively tripled the total color volume occupied by cotinga carotenoid plumages by creating entirely new purple-violet perceptual colors.

We further compared the color space distribution of the cotinga carotenoid reflectance spectra to reflectance spectra from a diverse sample of 118 carotenoid plumage patches from 40 avian species from 22 families from across Aves (including all non-cotinga species with carotenoid plumage coloration from Stoddard and Prum 2011) (Fig. 5e). The colors of the cotinga methoxy-ketocarotenoid plumages constitute an extension of the total distribution carotenoid-based plumages into an entirely new region of the color space that is unoccupied by any other known birds (Fig. 5e). This region of perceptual color space lies above the rest of the carotenoid gamut, and is a achieved specifically by the highly purple plumages of *Cotinga* and *Porphyrolaema,* and the pink plumage of *L. streptophorus,* which are all achieved with the cotingin and 2,3-didehydropompadourin. In addition, the rhodoxanthin-pigmented red crown of *P. carnifex* lies near to the red vertex of the tetrahedron outside the volume achieved by all other birds in the sample. Thus, this patch constitutes the most saturated, red carotenoid plumage known in birds.

These quantitative comparisons of cotinga plumage colors, as seen by the birds themselves, document that the metabolic novelties of various cotinga lineages have allowed them to produce new carotenoid molecules and deposit them in their feathers in ways that create distinct, new color stimuli that other groups of birds are unable to produce.

Phylogenetic evolution of metabolic pathways

The diversity of cotinga feather carotenoids documented here and higher level phylogenies for the group (Prum et al. 2000; Ohlson et al. 2007; Tello et al. 2009) provide an opportunity to analyze the evolution of carotenoid physiology within the clade. We did not observe circulating serum carotenoids in cotingas, so we cannot distinguish whether the plumage carotenoid composition is determined solely by metabolic modifications or by selective regulation of deposition into the feathers. It is possible that all cotinga species produce all of the carotenoids observed for

other physiological purposes, and that each species selectively deposit different ones into their feathers for coloration. However, previous experimental studies of carotenoid physiology in birds imply that systemic variations in metabolic capacities are crucial in determining carotenoid deposition in plumage (e.g. McGraw and Schuetz 2004). Relatively few species of cotingas have multiple plumage patches with distinct carotenoid colorations, which provide direct evidence of regulation of carotenoid deposition independent of metabolic modification. Several species of *Pipreola* fruiteaters have both lutein yellow belly patches, and orange or red throat with distinct carotenoid compositions. In most species of cotingas, variations among carotenoid plumage patches within a single cotinga plumage are likely to be produced by slight differences in total quantity of pigmentation rather than its composition.

A phylogenetic analysis of the presence or absence of carotenoid plumage pigmentation shows that carotenoid pigmentation is primitive to the cotinga family (Fig. 6a), and has been secondarily lost in the *Ampelion–Phytotoma–Doliornis–Zaratornis* clade, the *Lipaugus* pihas, and some combination of losses and rederivations in *Carpodectes* and *Conioptilon–Gymnoderus,* and the fruitcrows *Cephalopterus* and *Perissocephalus.*

Looking in greater detail at the inferred composition of the primitive carotenoid plumage, it is clear that unaltered dietary lutein is the sole carotenoid molecule in the primitive carotenoid plumage of cotingas (Fig. 6b). This condition has been retained unchanged from the ancestral state in the yellow plumages in four cotinga genera—*Ampelioides, Carpornis, Snowornis,* and *Tijuca*—and it has re-evolved in *Ampelion rufaxilla* and *Phibalura flavirostris.* Like the common ancestor of the cotingas, all these lineages appear to entirely lack the capacity for metabolic transformation of dietary carotenoids for deposition into their plumages (Fig. 7).

There have been several derived evolutionary changes in the composition of carotenoid plumages from the primitive state of lutein only: additions of zeaxanthin, β-cryptoxanthin, and β-carotene and their derivatives, or losses of the deposition of lutein and its derivatives. Evolutionary additions of zeaxanthin or its derivatives to plumage carotenoid composition have occurred numerous independent times in *Pipreola, R. peruviana, L. strepotophorus, Cotinga, Xipholena, Porphyrolaema,* and the fruitcrows *Haematoderus* and *Querula* (Fig. 6c). In most of these lineages, zeaxanthin derivatives have been merely added to lutein. However, in *R. peruviana* and the common ancestor of *Cotinga,* there was a simultaneous switch from primitive lutein-based plumages to entirely zeaxanthin-based carotenoid pigmentation. *L. streptophorus* is apparently an entirely novel origin of carotenoid pigmentation based entirely on zeaxanthin derivatives.

Even broader pigment compositions have evolved in other lineages: two evolutionarily independent origins of the use of β-cryptoxanthin in *R. peruviana* and *Xipholena*, and two of β-carotene in *Xipholena* and in the *Haematoderus-Querula* fruitcrows (Supplementary Figure S31).

Next, we can examine the evolution of metabolic modifications of plumage carotenoids. Again, we have only examined the expression of carotenoids in the plumage, and do not have direct physiological data on whether these same modifications occur for other physiological reasons within these species, and these phylogenetic patterns reflect the evolution of deposition and not metabolic capacity.

The use of ketocarotenoid pigments resulting from C4-oxygenation ($K_1 > 0$) has evolved between four and ten times independently (Fig. 7a): once each in the ancestor of red *Pipreola* species, in the genus *Rupicola,* in *L. streptophorus,* and at least once in the common ancestor of *Cotinga, Xipholena*, *Porphyrolaema, Cotinga, Haematoderus, Querula* and *Pyroderus* with several subsequent losses. Alternatively, the last hypothesized origin could be explained by as many as five independent origins and four evolutionary losses. All of these genera except *Pipreola* and *Pyroderus* have evolved to use methoxy-ketocarotenoids in their plumages, which require C3-O-methylation ($K_2 > 0$) (Fig. 7b). This phylogenetic distribution requires between seven independent origins and three independent origins with four evolutionary losses. All of these same lineages except *Rupicola* have evolved the capacity for C2–C3 didehydrogenation ($K_3 > 0$), requiring between six evolutionary origins and two origins with four losses (Fig. 7c).

The use of β-hydroxyl dehydrogenation to produce canary xanthophyll A or canary xanthophyll B (via C3′-hydroxylutein) ($K_4 > 0$) has evolved in *Procnias* bellbirds, the ancestor of *Phoenicircus* and *Rupicola,* and in *H. militaris* and *P. scutatus* (Supplementary Figure S32). ε-hydroxyl dehydrogenation ($K_5 > 0$) has likely also evolved in *Phoenicircus,* and possibly also in *Procnias* and *Haematoderus*. In *Haematoderus,* it is ambiguous whether ε-hydroxyl dehydrogenation is required for production of rhodoxanthin, or whether canary xanthophyll B is derived from zeaxanthin by β-hydroxyl dehydrogenation alone. In both *Phoenicircus and Haematoderus,* canary xanthophyll B is entirely converted to rhodoxanthin by subsequent C6-hydroxylation ($K_6 > 0$), and retro conversion ($K_7 > 0$) (Supplementary Figure S32). Hudon et al. (2007) present evidence from an analysis of a mutant and normal Pintailed Manakin that rhodoxanthin is produced metabolically from zeaxanthin via 3-hydroxylutein and canary xanthophyll B (Fig. 3). We hypothesize that rhodoxanthin in *Phoenicircus* is more likely to be produced from lutein. However, the presence of rhodoxanthin in a 3 to 1 ratio with pipraxanthin in the manakin *Ilicura militaris* indicates

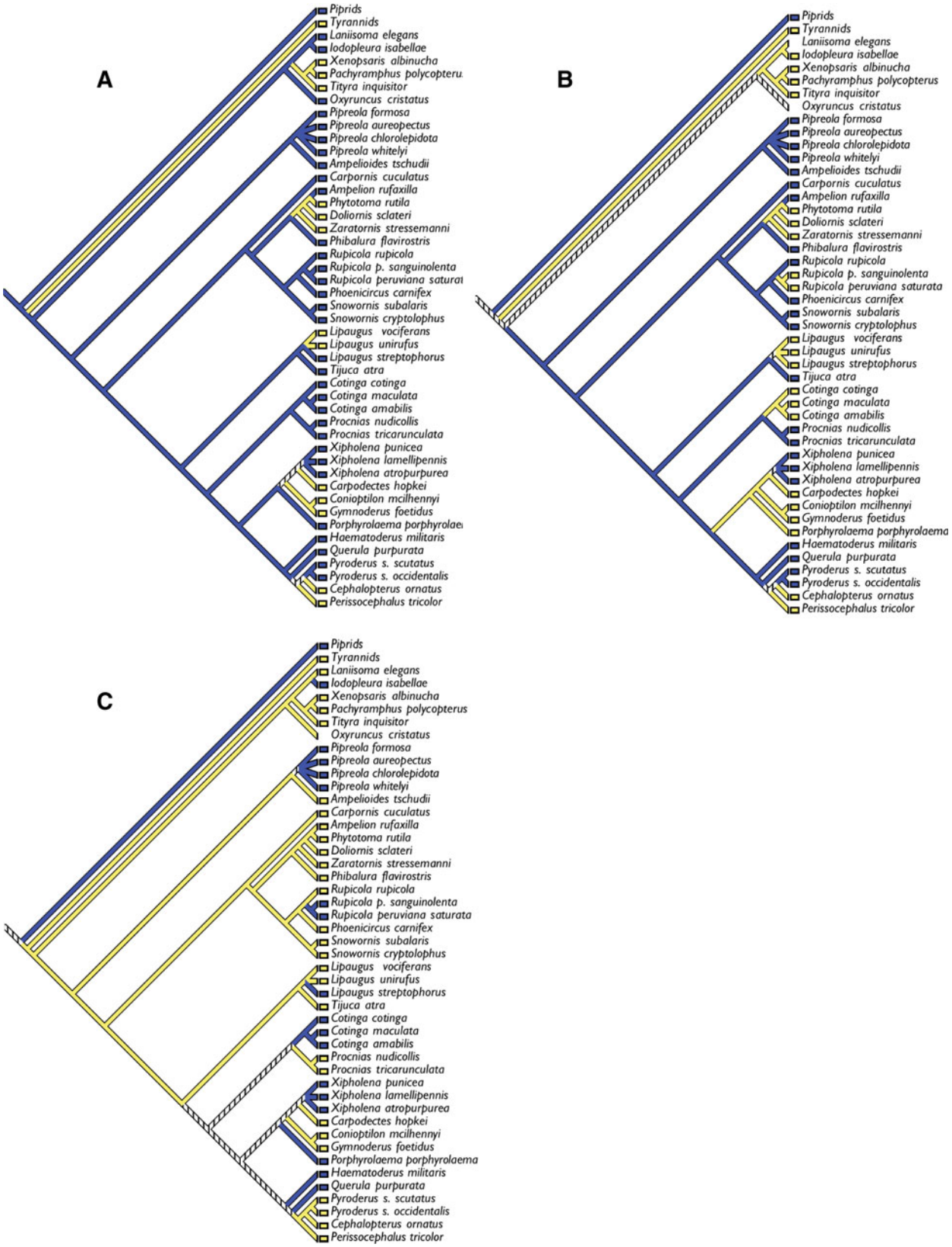

Fig. 6 Phylogenetic hypotheses for the evolution of carotenoid deposition, and lutein and zeaxanthin usage in plumages of cotingas and their outgroups. **a** Carotenoid plumage pigmentation (in *blue*), **b** plumage pigmentation using lutein and its metabolic derivatives (in *blue*), and **c** plumage pigmentation using zeaxanthin and its metabolic derivatives (in *blue*), based on a phylogeny by Tello et al. (2009)

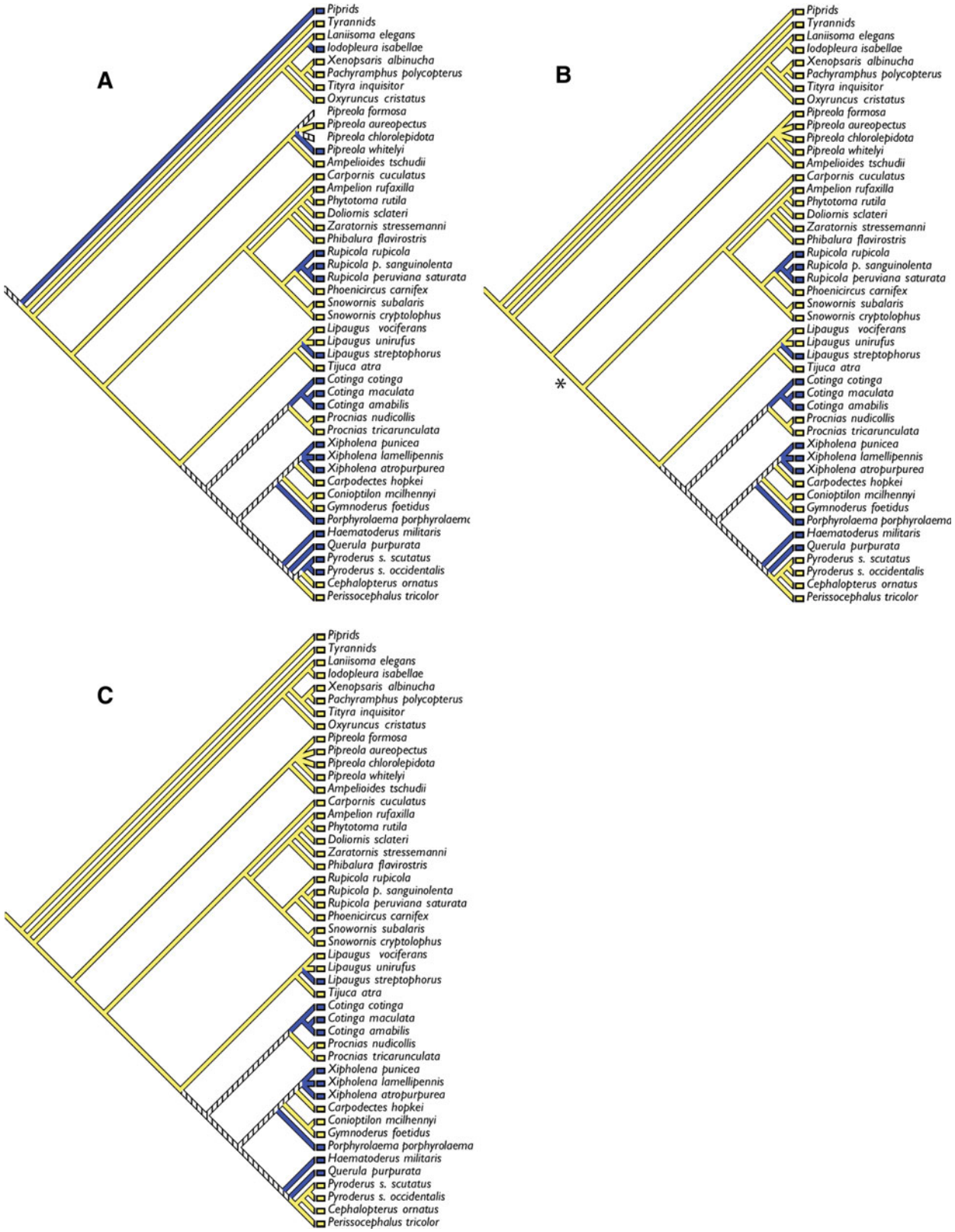

Fig. 7 Phylogenetic hypotheses for the evolution of metabolic reactions for the transformation of dietary precursor carotenoids in cotingas. **a** C4-oxygenation, $K_1 > 0$ (in *blue*); **b** C3-O-methylation, $K_2 > 0$ (in *blue*); and **c** C2–C3 didehydrogenation $K_3 > 0$ (in *blue*). An *asterisk* in **b** marks the most recent lineage in which a single evolutionary origin of C3-O-methylation could have occurred

that the final steps of rhodoxanthin synthesis have evolved to be more efficient in *Phoenicircus* than in *Ilicura* (i.e. K_6 and K_7 are greater in *Phoenicircus* than in *Ilicura*).

Discussion

An integrative comparative analysis of cotinga plumage carotenoid composition documents the dynamic evolution of the diversity of dietary precursor utilization, metabolic transformation of these dietary precursors, and visual color stimuli created by carotenoid pigments in this diverse Neotropical radiation. The methoxy-ketocarotenoids that we previously found in *X. punicea* (LaFountain et al. 2010) have been identified here in eleven additional species of cotingas within six additional genera. All of the six methoxy-ketocarotenoids found in *X. punicea* have here been found in other cotinga genera. In addition, we identified a new molecule—3′-hydroxy-3-methoxy-β,β-carotene-4-one, or rupicolin—in the Andean Cock-of-the-Rock, *R. peruviana* (Figs. 2, 4a).

Using the known carotenoid physiology pathways from other birds and vertebrates (Prager et al. 2009; Stradi et al. 1995, 1996, 2001; Andersson et al. 2007; Hudon et al. 2007), we have proposed metabolic pathways for the derivation of cotinga carotenoid molecules from common dietary precursors lutein, zeaxanthin, β-cryptoxanthin, and β-carotene. These pathways also predict the possible existence of yet undiscovered methoxy-carotenoids—including 3′-hydroxy-3-methoxy-canthaxanthin, which is a product of C3-O-methylation of astaxanthin (Fig. 3), and 2,3-didehydro-brittonxanthin which would be produced by C2,3-didehydrogenation of brittonxanthin (Fig. 4a).

Phylogenetic analysis of variation in plumage composition and the metabolic capacities required to produce the observed plumage pigments documents a dynamic pattern in the evolution of the utilization of different precursors (Figs. 6 and S31) and the metabolic transformations of these dietary molecules (Figs. 7 and S31). These evolutionary models require seven evolutionary events to describe the distribution of plumage methoxy-carotenoids ($K_2 > 0$); i.e., somewhere between seven independent origins and three origins with four subsequent losses. Given that the metabolic capacity for C3-O-methylation of ketocarotenoids is so far unique to these cotingas, it appears more likely that there was a single evolutionary origin of this enzymatic capacity in the most recent common ancestor of *Rupicola* and *Xipholena* (asterisk in Fig. 7b).

In addition to the fundamental role of carotenoid molecular diversity in producing plumage color variation of the cotingas, it is likely that the binding of the carotenoid molecules to feather β-keratin protein plays an additional role in the production of plumage color diversity. Stradi et al. (1995) demonstrated that yellow and red plumage patches in *Carduelis carduelis* are produced by the same mixture of canary xanthophylls, and implied the differences between two plumage colors involves variation in the conjugation of the same molecules. Recently, we have used resonance Raman spectroscopy to investigate variation in binding of canthaxanthin by feather proteins which results in the bright red of the Scarlet Ibis (*Eudocimus ibis*), the orange-red of the Summer Tanager (*Piranga rubra*), and the violet purple of the White-browed Purpletuft (*Iodopleura isabellae*) (Mendes-Pinto et al., in review). LaFountain et al. (2010) confirmed earlier findings (Völker 1952; Schmidt 1956) that the burgundy, methoxy-ketocarotenoid plumage coloration of *X. punicea* is both pressure and temperature sensitive, and that the pigments are orange in solvent solution. Similar observation contributed to their being dismissed as more conventional ketocarotenoids by earlier workers (Völker 1952; Schmidt 1956; Mattern and Völker 1955). In this data set, we find that the deeply purple plumage of *P. porphyrolaema* is nearly identical in carotenoid composition to the rosy pink plumage patches of the *L. streptophorus*—a mixture of cotingin and 2,3-didehydro-pompadourin. This finding further confirms the crucial role for the binding of methoxy-carotenoids to feather proteins in the production of plumage coloration diversity. Given that methoxy-carotenoids are not highly distinct in absorbance in solution from other ketocarotenoids, it appears that the function of the methoxy-groups of these molecules may be their in situ binding properties to proteins within the feather.

The outstanding diversity of cotinga methoxy-ketocarotenoids is achieved by the application of the same three enzymatic modifications: (1) C4-oxygenation (2) C3-O-methylation, (4) C2,3-didehydrogenation to three different molecules derived from three carotenoid precursors—lutein, zeaxanthin, and β-cryptoxanthin. The 3,3′-hydroxyl-β,β-ring structure of zeaxanthin provides two symmetric opportunities for these modifications on either ends of the molecule, giving rise to greater number of possible outcomes, whereas the restriction of 3-hydroxyl-β-ring structure to one side of lutein and β-cryptoxanthin results in additional chemically distinct metabolic products.

These comparative data document the contribution of the evolution of carotenoid precursor composition and metabolic transformations to plumage carotenoid composition. For example, the plumage carotenoid content of the two subspecies of *P. scutatus* examined were based on strikingly different dietary precursor compositions yet they had very similar K values. In contrast, the highly similar precursor carotenoid compositions of the plumages of *H. militaris* and *Q. purpurata* are combined with strong differences in the values of K_4 to K_7 to produce quite distinct profiles of total carotenoid content. In perhaps most

striking example, the clade including two species of *Phoenicircus*, *Rupicola rupicola* and *R. peruviana* constitutes a startling evolutionary radiation in plumage carotenoid content. Despite their close phylogenetic relationship and the fact that brilliant male carotenoid plumage coloration is shared among them from their common ancestor, they show none of their eight pigments in common other than lutein, which is found in substantial quantities in *R. rupicola* and as a trace in *P. carnifex.* So, avian plumage carotenoid composition can evolve both by evolutionary change in the composition of precursors utilized and in the metabolic modifications made to them, and evolution of both features can be very dynamic.

For most cotinga species examined, the physiological production of plumage carotenoid composition can be modeled accurately from the relative composition of precursors and the estimated rates of enzymatic modifications—i.e. the K_1 through K_7 values (Table 3). *X. punicea* provided three independent estimates of K_3, and provided highly concordant answers. However, there were a few deviations from this simple model that imply additional physiological processes are occurring in some species. For example, in *R. peruviana,* only zeaxanthin, adonixanthin and rupicolin are present (both of the latter are derived from the former; Fig. 4a). Given the values of K_1 and K_2 required to explain the relative frequencies of these three pigments, some additional physiological events are required to explain the absence of the production of astaxanthin, pompadourin, etc. This result documents that further study of avian carotenoid metabolism would yield interesting and undescribed new details.

Even the cotinga species that utilize methoxy-ketocarotenoids in their plumage show extensive diversity in plumage pigment composition. In *Rupicola,* the two species use entirely different precursors—lutein versus zeaxanthin—but both species produce a single methoxy-ketocarotenoid as a moderate component of a mixture of other pigments. By contrast, *Cotinga* plumages contain only cotingin, and *Porphyrolaema* and *L. streptophorus* contain mostly cotingin and some 2,3-didehydro-pompadourin. These pigments imply that these species are restricted to the metabolic modification of zeaxanthin precursors, and that the efficiency of C4-oxygenation and C3-O-methylation is sufficiently high to entirely convert all precursor molecules into the last two molecules in the pathway. The production of cotingin requires six metabolic changes of dietary zeaxanthin employing three different enzymes, making it among the most extreme examples of metabolic carotenoid modification in birds (McGraw 2006).

Querula and *Haematoderus* display other unique combinations of methoxy-ketocarotenoids—brittonxanthin, pompadourin, 2, 3-didehydro-pompadourin, xipholenin, and 2,3-didehydro-xipholenin—which are metabolic derivatives of β-cryptoxanthin, zeaxanthin, and lutein, respectively. The absence in *Querula* and *Haematoderus* of cotingin, which is found in *Cotinga, Porphyrolaema*, and *L. streptophorus*, implies that *Haematoderus* and *Querula* have lower efficiency of C2–C3 didehydrogenation (K_3) which prevents the complete conversion of zeaxanthin beyond 2,3-didehydro-pompadourin in that pathway.

Lastly, variation among the species of *Xipholena* implies additional evolution in the relative efficiencies of various metabolic enzymes. *Xipholenas* pigments are derived from four dietary precursors—zeaxanthin, lutein, β-cryptoxanthin, and β-carotene. The metabolic modifications of these molecules are efficient enough that none of these precursors are deposited unmodified into the plumage, but not so efficient that subsequent reactions go to complete modification. The result is extensive carotenoid diversity within the plumage. *X. lamellipennis* and *Xipholena atropurpurea* have similar carotenoid composition to one another—canthaxanthin, pompadourin, xipholenin, and 2,3-didehydro-pompadourin—but both lack astaxanthin, 2,3-didehydro-xipholenin, and cotingin found in *X. punicea*. The differences require: (1) the absence of concentration and utilization of β-carotene as a precursor, and (2) a lower efficiency of C2–C3 didehydrogenation in *X. lamellipennis* and *Xipholena atropurpurea* compared to *X. punicea.*

A large literature has focused on the evolution of plumage carotenoid pigments as honest signals of condition and quality (reviewed in Hill and McGraw 2006a, b). Given the unique, derived features of cotinga plumage carotenoid physiology, the question arises whether these features are consistent with honest signaling theory. Signal honesty is enforced by the costs of the signal. However, tropical fruit diets of cotinga strongly suggest that carotenoids are not limiting. It would be hard to imagine these species getting enough calories to survive without getting excess carotenoids to pigment their plumages. Further, we have shown that plumage carotenoid composition in *X. punicea* did not vary significantly between a >100-year-old museum skin and a modern zoo bird (LaFountain et al. 2010), so cotinga plumage carotenoid composition appears to be rather robust to variation in diet. If cotinga carotenoids are hypothesized to be honest signals of genetic quality or condition, such signals would likely have to gain their quality or condition correlation through some other mechanism than dietary limitation or cost.

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