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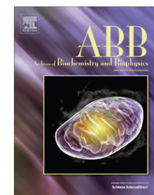

Carotenoids from the crimson and maroon plumages of Old World orioles (Oriolidae)

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ABSTRACT

Recent analyses of the orange, red, and purple plumages of cotingas (Cotingidae) and broadbills (Eurylaimidae) revealed the presence of novel carotenoid molecules, suggesting that the diversity of pigments and the metabolic transformations they undergo are not yet fully understood. Two Old World orioles, the Black-and-Crimson Oriole *Oriolus cruentus*, and the Maroon Oriole *Oriolus traillii*, exhibit plumage colors that are similar to those of some cotingas and broadbills. To determine if these oriole plumage colors are produced by the same carotenoids or with other molecules, we used high-performance liquid chromatography (HPLC), mass spectrometry, and chemical analyses. The data show that the bright red feathers of *O. cruentus* contain a suite of keto-carotenoids commonly found in avian plumages, including canthaxanthin, adonirubin, astaxanthin, papilioerythrinone, and α-doradexanthin. The maroon feathers of *O. traillii* were found to contain canthaxanthin, α-doradexanthin, and one novel carotenoid, 3′,4-dihydroxy-ε,ε-carotene-3-one, which we have termed "4-hydroxy-canary xanthophyll A." In this paper we propose the metabolic pathways by which these pigments are formed. This work advances our understanding of the evolution of carotenoid metabolism in birds and the mechanisms by which birds achieve their vivid plumage colorations.

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Introduction

A number of birds display[1] vivid plumage resulting from pigmentary or structural colors, or combinations of both. The bright yellow, orange, red and even purple plumage colors are produced by carotenoid pigments. Birds ingest carotenoids in their diets and deposit them in plumage either as unaltered dietary compounds, or after various metabolic, chemical modifications [1].

Recent studies of brightly colored plumage from various avian species have revealed novel carotenoid structures. For example, pigment analyses of orange, red, and purple feathers of cotingas (Cotingidae) have revealed that these colors are produced by seven previously unknown methoxy-ketocarotenoids [2,3]. Furthermore, we have also found that maroon, pink, and lavender plumage colors in the Asian broadbills (Eurylaimidae) are produced by another novel carotenoid [4]. Thus, it appears that avian plumage carotenoid diversity has not been sufficiently well surveyed, and that there may be additional, previously unknown molecules awaiting description. In particular, we were interested in whether the deep maroon or crimson plumage colors that are produced by distinctive, metabolically modified carotenoids in cotingas (e.g. *Xipholena*, *Querula*) and broadbills (e.g. *Cymbirhynchus*, *Eurylaimus*) are produce by similar pigments in other bird species.

We investigated the pigment composition in the plumage of two closely-related species of Old World orioles (Oriolidae: Passeriformes): the Black-and-Crimson Oriole *Oriolus cruentus*, and the Maroon Oriole *Oriolus traillii* (Fig. 1). The males of both species have extensively black plumage with patches of deep crimson red on the lower breast and wing (*O. cruentus*), or deep maroon on the entire underparts, back, rump, and tail (*O. traillii*). Females of both species have dark or drab plumage with obvious carotenoid patches.

Old World orioles include 33 species that are found in tropical and warm temperate latitudes of Asia, Europe, Africa, and eastern Australia [5]. Orioles are omnivorous, and have a substantial amount of fruit in their diets [5]. Most species of *Oriolus* have bold black and bright yellow plumages. The hue of yellow is surprisingly uniform among different *Oriolus* species. In two species, the yellow plumage colors of the Golden Oriole (*O. oriolus*) and the Black-hooded Oriole (*O. xanthornus*) are produced by a combination lutein and zeaxanthin [6]– two molecules produced by plants that

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[1] *Abbreviations used:* HPLC, high-performance liquid chromatography; APCI, atmospheric pressure chemical ionization; $NaBH_4$, sodium borohydride; MTBE, methyl tert-butyl ether.

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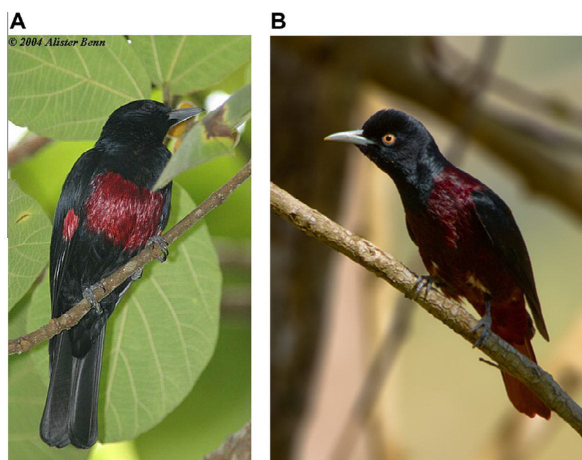

Fig. 1. Photographs of (A) the Black-and-Crimson Oriole *Oriolus cruentus*; and (B) the Maroon Oriole *O. traillii traillii*. Photo credits: (A) Alister Benn; (B) Subharghya Das. Reproduced with permission.

are common in avian diets. Although many species of *Oriolus* have bright red bills, implying that they may be able to metabolically modify dietary carotenoids to form red ketocarotenoids, they do not deposit these molecules in their plumages.

Oriolus cruentus and *O. traillii* are two closely related oriole species that are found in sub-tropical and tropical habitats in Southeast Asia [7], and they are the only *Oriolus* species with red plumage. The similarity of these deep crimson and maroon-brown colors to plumages of certain cotingas and broadbills motivated us to identify the pigments responsible for these vivid colors and to elucidate the metabolic pathways required for their production. Ultimately, understanding the variety of carotenoid molecules used by birds to achieve various plumage colors will help us to gain insights into the evolution of the metabolic pathways that are used to produce them and the mechanisms by which carotenoids are spectrally tuned in feathers.

Methods

The crimson breast feathers of the Black-and-Crimson Oriole, *O. cruentus*, and the maroon breast feathers of the Maroon Oriole, *Oriolus traillii traillii*, were obtained from the Yale Peabody Museum collection in New Haven, CT. The *O. cruentus* feathers were obtained from specimen number YPM 63643, which was collected in Malaysia in 1952. The *O. traillii* feathers were obtained from specimen YPM 64070, which was collected in Thailand in 1957. Feathers of the highest visual quality were selected.

Pigments were extracted from the feathers by soaking in acidified pyridine at 90 °C for three hours according to the methods outlined in LaFountain et al. [2]. Following three hours of heating in the presence of acidified pyridine, feathers from both species remained orange in color. It is likely that this residual pigmentation is due to the presence of melanins, which would not be extracted by the heated acidified pyridine method [1,8,9].

Extracts were analyzed by HPLC as previously described [2,3]. Analysis was conducted using a Waters 600E/600S multi-solvent delivery system equipped with a Waters 2996 detector. The column was a Phenomenex Luna 5 μ silica column, and the mobile phase protocol consisted of a linear gradient from 90% hexane, 10% acetone to 80% hexane, 20% acetone over a period of 40 min, followed by 20 min of isocratic delivery of 80% hexane and 20% acetone. The flow rate was 1.5 mL/min. Bona fide astaxanthin and canthaxanthin, obtained as gifts from Roche, were analyzed using the same HPLC protocol for comparison.

The *O. cruentatus* peaks numerically labeled 1–6 in Fig. 2A and the *O. traillii* peaks labeled 1, 3, and 4 in Fig. 2B were collected, dried under nitrogen gas, re-dissolved in methanol, and subjected to mass spectral analysis on an Applied Biosystems API 2000 mass spectrometer using an atmospheric pressure chemical ionization (APCI) probe in both positive and negative mode. The samples were injected by direct infusion at a flow rate of 40 μL/min. The injection solvent was methanol.

Peaks collected from the HPLC chromatograms at 8.9 and 11.2 min from *O. cruentus* and at 12.6 and 17 min from *O. traillii* feather extracts were treated with sodium borohydride ($NaBH_4$) in order to determine the number of carbonyl groups present on the molecules. The samples were evaporated to dryness, re-dissolved in methanol prior to $NaBH_4$ treatment, and monitored for spectral changes on a Varian Cary UV/Vis spectrophotometer following the addition of 100 μg of sodium borohydride. Additional sodium borohydride was added as needed to achieve the characteristic absorption spectrum of either a β- or α-carotene chromophore [10], which indicate that any conjugated carbonyls have been reduced. The sample was then taken up in methyl tert-butyl ether (MTBE)/water (3:1, v/v) to stop the reaction. The pigmented MTBE layer was collected, dried under nitrogen gas, and then re-dissolved in the HPLC injection solvent, which was 14% acetone in hexane (v/v). The HPLC chromatogram of the reduction product was compared to that of the untreated pigment. The masses of the HPLC-pure reduction products of peaks isolated at 8.9 and 11.2 min from *O. cruentus*, and 12.6 and 17 min from *O. traillii* feather extracts were also determined by mass spectrometry.

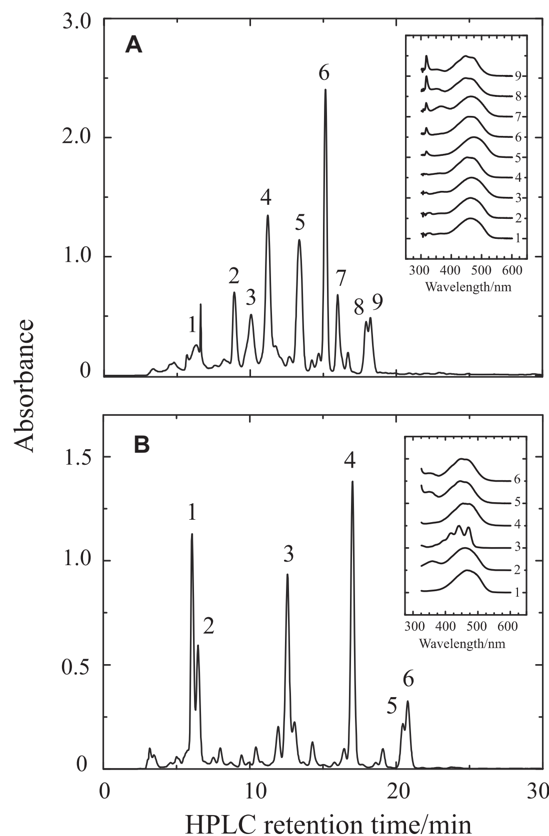

Fig. 2. (A) HPLC chromatogram of *O. cruentus* feather extract, detected at 460 nm; with inset of UV/Vis absorption spectra of HPLC peaks from *O. cruentus* feather extract as recorded by the photodiode array detector in the mobile phase solvent; (B) HPLC chromatogram of *O. traillii* extract, detected at 470 nm with inset of UV/Vis absorption spectra of HPLC peaks from *O. traillii* feather extract as recorded by the photodiode array detector in the mobile phase solvent.

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Table 1
HPLC retention time, mass percentage, and parent ion mass values for pigments isolated from *O. cruentus* and *O. traillii* feathers (n.d. = not determined).

Peak no.	Carotenoid assignment	HPLC retention time (min)	Mole percent of total carotenoids	Parent ion mass (m/z)
O. cruentus				
1	Canthaxanthin	6.3	5	564
2	Adonirubin	8.9	9	580
3	Adonirubin cis-isomer	10.1	11	n.d.
4	Papilioerythrinone	11.2	18	580
5	Astaxanthin	13.4	20	596
6	α-Doradexanthin	15.1	20	582
7	Astaxanthin cis-isomer	16.0	8	n.d.
8	α-Doradexanthin cis-isomer	17.9	4	n.d.
9	α-Doradexanthin cis-isomer	18.2	5	n.d.
O. traillii				
1	Canthaxanthin	6.1	20	564
2	Canthaxanthin cis-isomer	6.4	12	n.d.
3	4-Hydroxy-canary xanthophyll A	12.6	34	582
4	α-Doradexanthin	17.0	23	582
5	α-Doradexanthin cis-isomer	20.4	4	n.d.
6	α-Doradexanthin cis-isomer	20.7	7	n.d.

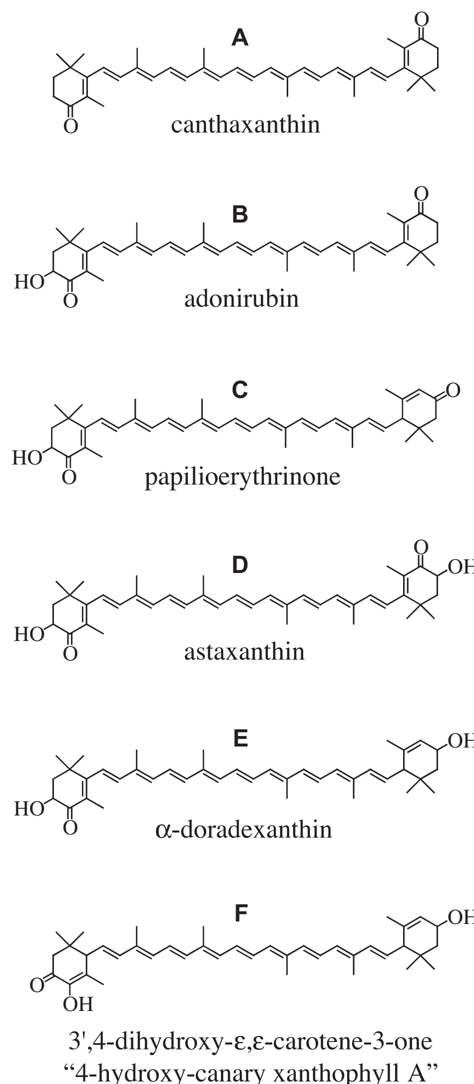

Fig. 3. Assigned structures of *O. cruentus* and *O. traillii* carotenoid pigments. (A–E), found in *O. cruentus*, and (A, E, and F) are found in *O. traillii*.

Results

Oriolus cruentus

HPLC analysis of the crimson *O. cruentus* feather extract resolved nine major peaks (Fig. 2A) all of which had broad spectra indicative of carotenoids with at least one conjugated carbonyl group. The results of the mass spectral analysis of peaks 1–6 are given in Table 1, and described in more detail below. Reduction by sodium borohydride and subsequent HPLC analysis of the reduction products were conducted on peaks collected at 8.9 min (Fig. 2A, peak 2) and 11.2 min (Fig. 2A, peak 4) to assess the number of carbonyl groups present in the carotenoid structure.

Peak 1, observed at 6.3 min in Fig. 2A, showed a broad absorption spectrum with essentially no vibronic structure which is typical of a carotenoid with two conjugated carbonyls. It also had an HPLC retention time consistent with that of a bona fide standard of canthaxanthin. The mass was found to be 564 m/z. Based on these observations, the peak was identified as canthaxanthin (Fig. 3A) without further analysis.

Peak 2, observed at 8.9 min in the HPLC chromatogram (Fig. 2A), displayed a similar absorption spectrum to that of peak 1, and was found to have a mass of 580 m/z. Treatment of the pigment with borohydride resulted in a blue-shift consistent with two carbonyls in conjugation, and yielded a β-carotene-like chromophore. HPLC analysis of the reduction product resulted in two peaks. These peaks eluted from the HPLC column later than the original peak, which is indicative of increased polarity upon reduction. The two observed peaks represent mono- and di-reduced products, indicating that the pigment contains multiple carbonyls. These two reduction products were analyzed by mass spectrometry and were confirmed to have a mass of 584 m/z, consistent with the reduction of 2 carbonyls. This pigment is therefore identified as adonirubin (Fig. 3B).

Peak 3, observed at 10.1 min in Fig. 2A, is identified as a cis-isomer based on the cis-band observed at 365 nm in the absorption spectrum. The absorption spectrum is broad and featureless similar to adonirubin, and is therefore likely a cis-isomer of this molecule which was generated as an artifact during the extraction procedure.

The peak observed at 11.2 min (Fig. 2A, peak 4) displayed a broad absorption spectrum with a small amount of vibronic resolution characteristic of a carotenoid having one conjugated carbonyl. The mass of this peak was found to be 580 m/z, with MS/MS fragments including M-18, M-92, 233, 202, 167, 137 m/z. All of these fragments are commonly associated with the fragmentation of either zeaxanthin or astaxanthin in negative ion mode [11], indicating that one of the rings on this pigment is likely a 3-hydroxyl-4-keto-β-ring. The 16 mass unit difference between the mass of astaxanthin and peak 4 suggests that the second ring has only one oxygen functional group. Treatment of this pigment with sodium borohydride resulted in a blue-shift consistent with one carbonyl in conjugation and yielded an α-carotene-like chromophore. HPLC analysis of the reduction product shows multiple peaks of increased polarity relative to the original, with all peaks having the same absorption spectra and thus indicating that at least one carbonyl group exists out of the π-electron conjugation and that the molecule is not symmetric. Based on these observations, this peak is identified as papilioerythrinone

(Fig 3C). Papilioerythrinone is a well-known, but not very common, avian carotenoid [1].

Peak 5 (13.4 min, Fig. 2A) displayed the broad absorption spectrum characteristic of a carotenoid with two conjugated carbonyls, and a retention time the same as that of a bona fide astaxanthin standard. The mass was found to be 596 m/z, which is also consistent with astaxanthin. This peak is therefore identified as astaxanthin (Fig. 3D).

Peak 6 (15.1 min, Fig. 2A) has a similar broad absorption spectrum to that seen from peak 4 and which is characteristic of a carotenoid having one conjugated carbonyl group. The mass of this peak was found to be 582 m/z in positive mode and 564 m/z in negative mode, the latter representing the molecular ion less one hydroxyl group. MS/MS of the pigment in negative mode yielded the following fragments: M-92, M-260, 233, 207, 167, 137. Several of these fragments, including M-92, 233, 167, and 137 m/z are associated with astaxanthin, indicating the presence of a 3-hydroxyl-4-keto-β-ring. Therefore, this peak is identified as α-doradexanthin (Fig. 3E).

Peaks 7, 8, and 9 (16.0, 17.9, and 18.2 min, respectively) all show prominent cis-bands in the vicinity of 350–360 nm, indicating that they are isomers of the more abundant pigments. Peak 7 has a broad absorption spectrum similar to that of astaxanthin and is retained in close proximity, and is therefore identified as an astaxanthin cis isomer. The absorption spectra of peaks 8 and 9 are similar to that of α-doradexanthin, and therefore these pigments are identified as α-doradexanthin cis-isomers.

The total carotenoid content of the feathers was composed of 29% α-doradexanthin, 28% astaxanthin, and ~18% papilioerythrinone with smaller quantities of the other molecules (Table 1).

Oriolus traillii

HPLC analysis of the maroon breast feathers of *O. traillii* revealed six separate major peaks (Fig. 2B). All other peaks were likely to be cis-isomers and other minor components, and were not examined.

Peak 1 (6.1 min, Fig. 2B) had a broad, featureless absorption spectrum with a λ_{max} at ~473 nm in ethanol (Fig. 2B, trace 1). Along with the HPLC retention time of 6.1 min, this observation is consistent with the carotenoid being canthaxanthin. This identification was confirmed by mass spectral analysis which revealed a mass of 564 m/z. Peak 2 had a similar absorption spectrum with a cis band at ~350 nm (Fig. 2B, trace 2), and is therefore identified as a cis-isomer of canthaxanthin.

The absorption spectrum of peak 3 showed a high degree of vibronic structure in ethanol (%III/II = 76.5) [12] indicating that this molecule does not contain a carbonyl in conjugation with the π-electron chain. The absorption spectrum having a λ_{max} at 441 nm closely matches those reported for canary xanthophyll A and B [10], and therefore suggests a carotenoid with 9 conjugated carbon–carbon double bonds and an ε,ε-carotene chromophore. As expected from the inferred absence of conjugated carbonyls, reduction by sodium borohydride did not affect the absorption spectrum. However, HPLC analysis of the reduction product did show multiple peaks of a more polar nature than the unreacted pigment, indicating that the molecule does have at least one carbonyl group located out of conjugation.

The mass of the untreated sample was found to be 582 m/z in positive mode and 564 m/z in negative mode. MS/MS analysis of the peak in positive mode revealed a fragment with a mass of 565 m/z, consistent with the loss of one hydroxyl (M-18), which confirms that the 564 m/z mass observed in negative mode likely represents the molecular ion less one hydroxyl group. The fragments observed in negative mode also included: M-33, M-152, 219, 164, 152, and 137. M-33 and 137 are fragments commonly associated with both lutein and zeaxanthin [11], indicating that one of the rings may be either a 3-hydroxy-β- or a 3-hydroxy-α-ring.

The M-152 ion observed in the MS/MS spectrum of this pigment is suggestive of a carotenoid with saturation of the C7, C8 double bond [13]. Stradi et al. [13] previously reported the presence of 7,8-dihydro-xanthophylls in woodpeckers, and termed the pigments "picofulvins". However, the absorption spectrum of the 7,8-dihydro-carotenoid, 7,8-dihydro-β-carotene [10] displays a λ_{max} at 429 nm, which is 12 nm blue-shifted compared to the spectrum from this unknown pigment. Therefore, we can conclude that the unknown pigment is not a 7,8-dihydro-carotenoid and does have an unsaturated C7, C8 bond, and thus we can conclude that the $n = 9$ conjugated chain extends from C6 to C6′.

Based the mass spectral data, the results of the borohydride reaction, and similarities of this pigment to the canary xanthophylls, we propose that the structure of this pigment is that of 3′,4-dihydroxy-ε,ε-carotene-3-one (Fig. 3F). This structure is similar to a known avian carotenoid, canary xanthophyll A, but has an additional hydroxyl group in the C4 position. Therefore, this pigment could also be referred to as "4-hydroxy-canary xanthophyll A". Previous experiments conducted using the same HPLC protocol revealed that canary xanthophyll A and B elute at 8 and 12 min (respectively) from the extract of female *Procnias tricarunculata* belly feathers [3]. A canary xanthophyll analog with hydroxyl groups in the C3′ and C4 positions would be expected to behave relatively more polar in nature than one which has a carbonyl in the C3′ position, so a longer retention time on a normal phase column (~15 min) is consistent with this assignment.

The absorption spectrum of peak 4 (Fig. 2B, trace 4) has a broad spectrum suggestive of one conjugated carbonyl. Upon borohydride reduction, the spectrum blue-shifts by ~13 nm, confirming the presence of one conjugated carbonyl, and sharpens to reveal an α-carotene-like chromophore. HPLC analysis of this reduction product reveals two peaks of nearly equal intensity that are more polar relative to the original unreduced peak.

Mass spectral analysis of peak 4 revealed a mass of 582 m/z in positive mode and 564 m/z in negative mode, as was observed in the mass spectrum of peak 3. MS/MS fragments in negative mode included: M-18, M-92, 233, 207, and 167. The same mass and the same five fragments were also observed in the MS/MS of peak 6 from *O. cruentus*. Based on these similarities and the results from the borohydride reduction, this peak is identified as α-doradexanthin.

Peaks 5 and 6 (Fig. 2B) have similar absorption spectra to peak 4, and characteristic cis-bands in the vicinity of 360 nm. Therefore, based on their close proximity to α-doradexanthin in the HPLC, and their spectral characteristics, these peaks are identified as cis-isomers of α-doradexanthin. These isomers were likely created during the extraction procedure.

The total carotenoid content of the feathers was composed of approximately one third each of canthaxanthin, α-doradexanthin, and 3′-dehydro-canary xanthophyll C (Table 1).

Discussion

Proposed metabolic pathways for the formation of the five pigments found in *O. cruentus* are presented in Fig. 4A–D, and for the three pigments found in *O. traillii*, in Fig. 4A, D, and E. These pathways represent the most likely series of transformations derived from the four known dietary carotenoids, β-carotene, β-cryptoxanthin, lutein and zeaxanthin [1] using metabolic transformations that have been previously reported in birds [1,14–16]. Tests for the existence of the enzymes responsible for these transformations would require labeling and/or dietary studies.

Two of the primary pigments in both species are α-doradexanthin and canthaxanthin (Table 1). α-Doradexanthin has been

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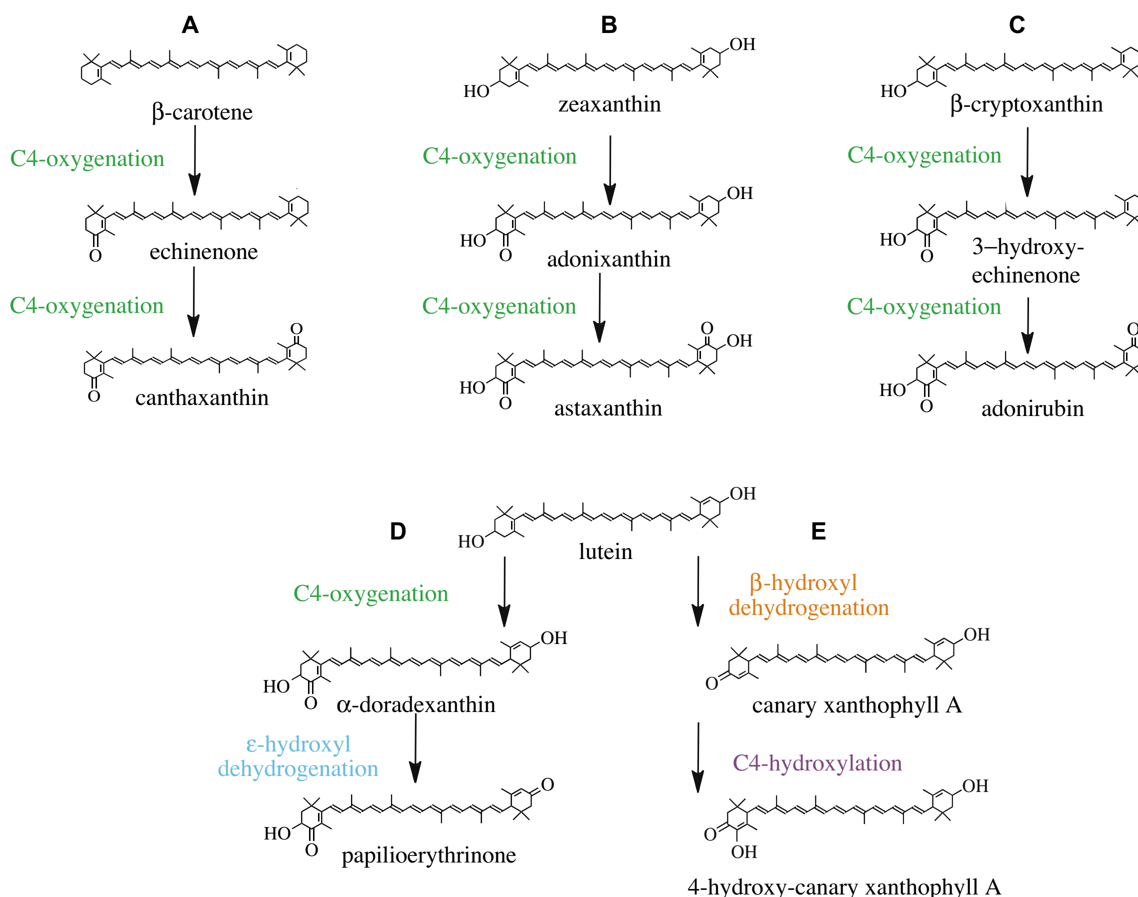

Fig. 4. Proposed metabolic pathway for the pigments isolated from *O. cruentus and O. traillii*: (A) canthaxanthin; (B) astaxanthin; (C) adonirubin; (D) α-doradexanthin and papilioerythrinone; and (E) 4-hydroxy-canary xanthophyll A. Enzymatic steps in these pathways have been previously presented in a compilation prepared by McGraw [1]. (A–D) occur in *O. cruentus*, and A, D, and E occur in *O. traillii traillii*.

reported in the in red plumage of multiple species of woodpeckers (Picidae) [1,17], and also within several families of perching birds (Passeriformes) [1,18]. It is easily derived from the abundant dietary carotenoid lutein through a single C4-oxygenation (Fig. 4D). Canthaxanthin is a common carotenoid found in avian plumage exhibiting a wide range of colors, including the red feathers of the Scarlet Ibis (*Eudocimus ruber*) [19,20], pink feathers of the flamingos (*Phoenicopterus ruber*, *P. chilensis*, and *P. minor*) [21], red feathers of manakins [22], purple axillary feathers of the White-browed Purpletuft (*Iodopluera isabellae*; Tityridae)[20], and several others [1,15,18,23,24]. *O. cruentus* was additionally found to contain the red ketocarotenoids astaxanthin and adonirubin, which are also relatively common in avian plumage [1,18].

Canthaxanthin, astaxanthin and adonirubin are all derived from the dietary carotenoids, β-carotene, zeaxanthin, and β-cryptoxanthin, respectively, via C4-oxygenation of both β-ring end groups. This process is widely accepted to occur in plumage and is attributed to an enzyme generally referred to as C4-oxygenase [1,13–16,18,25]. Papilioerythrinone, the fifth pigment detected in *O. cruentus*, is thought to be derived from α-doradexanthin, by the dehydration of the hydroxyl group on the C3′ position of the ε-ring (Fig. 4D) [1]. While the occurrence of papilioerythrinone in red plumage is less common, it has been reported within the order Passeriformes in the Eurasian Bullfinch (*Pyrrhula pyrrhula*) [24], and in two species of rosefinch, the Beautiful Rosefinch (*Carpodacus pulcherrimus*) and Pallas's Rosefinch (*Carpodacus roseus*) [1,17]. Additionally, we have recently found papilioerythrinone derivatives in the red and rosy plumage of four broadbill species [4].

O. trailli was found to contain two of the same pigments as *O. cruentus*–canthaxanthin and α-doradexanthin– which are derived from the dietary carotenoids, β-carotene and lutein, respectively (Fig. 4A and D). The absence of astaxanthin and adonirubin despite an evidenced ability to perform C4-oxygenation implies that *O. traillii* has either not ingested, accumulated, or transported the dietary carotenoids, zeaxanthin or β-cryptoxanthin.

The maroon plumage of *O. traillii* contains a third, novel pigment that we have named 4-hydroxy-canary xanthophyll A. This novel molecule is likely formed from the dietary precursor lutein via the intermediate canary xanthophyll A, as shown in Fig. 4E. It has been proposed that canary xanthophyll A is formed from lutein by dehydrogenation of the β-ring [1,14,15], and followed by the addition of a hydroxyl group to the C4 position of canary xanthophyll A to create 4-hydroxy-canary xanthophyll A.

In some bird species, canary xanthophyll A can also be metabolized into canary xanthophyll B by dehydrogenation of the ε-ring [1,3,14,25,26]. It has been postulated that two different enzymes are responsible for the dehydrogenation of a β-ring versus an ε-ring [3]. -Consequently, the lack of a specific enzyme, ε-hydroxyl dehydrogenase, would render the organism unable to produce canary xanthophyll B from canary xanthophyll A. The pigment composition of *O. traillii* feathers provides further support for the hypothesis that separate enzymes are required for dehydrogenation of β- versus ε-rings. The co-occurrence of α-doradexanthin

and canary xanthophyll A, and lack of canary xanthophyll B, provides good evidence that lutein was accumulated from the diet and metabolized, but that *O. traillii* is only capable of β-hydroxyl dehydrogenation.

It is very likely that the plumage of both species contains pheomelanins since the feathers retained a pale brownish-orange hue after heating in acidified pyridine. While the presence of melanins and carotenoids is common within an individual feather, it is rare for these pigments to be found in the same section of the feather [8]. This phenomenon has previously been reported in the red epaulets of the red-winged blackbird [8], and suspected in the red–orange feathers of the cotinga, *Pyroderus scutatus* [3] and in the red feathers of two species of fruit pigeons, *Ptilinopus magnificus* and *Ptilinopus pulchellus* [27]. It is not clear how the co-existence of carotenoids and melanins contributes to the observed colors of the feathers.

We have analyzed the plumage of a Maroon Oriole specimen from the nominate subspecies *O. traillii traillii*, which is found in the northern and western portions of the species distribution in southern China, the northern parts of Laos, Vietnam, Thailand, and Myanmar, and in the Himalayas. The body plumage of *O. traillii traillii* is deep maroon brown color. However, the subspecies of Maroon Oriole found in southern Laos and Vietnam (*O. traillii robinsoni)* and on Taiwan (*O. traillii ardens*) are more brilliant crimson, and closer in hue to *O. cruentus*. Further research on population level variation in the carotenoid content of these populations could yield further insights into the microevolution of carotenoid plumage composition.

Based on the current hypothesis of phylogeny for the Oriolidae [7], *O. cruentus* and *O. traillii* are members of restricted clade of four species. *O. cruentus* is sister to the *O. hosii* which is nearly all black with rufous undertails that are presumably produced by pheomelanin pigments. *O. traillii* is the sister species to *O. mellianus*, which differs in having pale whitish body plumage and a drabber maroon tail. Furthermore, according to the Jønsson et al. phylogeny [7], the bright yellow plumage was derived early in the history of the genus: i.e., in the most recent common ancestor of *O. xanthonotus* and *O. oriolus* (Clade II and Clade VI in Jønsson et al.).

Based on Stradi's [6] observation of lutein and zeaxanthin only in the yellow plumage of *O. oriolus* and *O. xanthornus*, it appears that the deposition of these unmodified dietary precursors is evolutionarily primitive to the production and use of ketocarotenoids in the *O. cruentus* and *O. traillii.* The capacity to metabolize dietary carotenoids via C4-oxygenation and to transport those products into the feathers likely evolved in the common ancestor of the *cruentus–traillii–hosii–mellianus* clade, and was lost in *O. hosii.* and *O. mellianus*. Subsequent to the origin of the capacity for C4-oxygenation, *O. cruentus* evolved to utilize four different dietary precursors, and the capacity for ε-hydroxyl dehydrogenation to produce papilioerythrinone from α-doradexanthin. In contrast, since common ancestry with *cruentus*, *O. traillii* evolved to utilize β-carotene and lutein derivatives alone, which were modified by C4-oxygenation (canthaxanthin and α-doradexanthin, respectively). Further, *O. traillii* evolved the novel capacity for β-hydroxyl dehydrogenation and C4-hydroxylation to produce 4-hydroxy-canary xanthophyll A from lutein.

Conclusion

The crimson feathers of *O. cruentus* contain a broad suite of keto-carotenoids that are commonly observed in red birds, including canthaxanthin, astaxanthin, adonirubin and α-doradexanthin, along with a fifth less-commonly observed pigment, papilioerythrinone. These five carotenoids are derived from the four avian dietary carotenoids, β-carotene, β-cryptoxanthin, lutein, and zeaxanthin, via two metabolic transformations, C4-oxygenation and ε-hydroxyl dehydrogenation. The maroon feathers of *O. traillii* contain the common ketocarotenoids, canthaxanthin and α-doradexanthin, as well as a third, novel carotenoid, 4-hydroxy-canary xanthophyll A. These carotenoids are derived from lutein and β-carotene by three metabolic transformations, C4-oxygenation, β-hydroxyl dehydrogenation, and C4-hydroxylation.

Comparing the pigment compositions of these two birds, it seems that *O. cruentus* is only able to perform C3-hydroxyl-dehydrogenation on the ε-ring, while *O. traillii* is only able to perform the same reaction on the β-ring. This finding provides evidence that specific, separate enzymes are required for C3-hydroxyl-dehydrogenation of β- and ε-rings.

These southeast Asian orioles produce crimson and maroon plumage colors with combinations of different carotenoid pigments than we have found the Neotropical cotingas (e.g., *Xipholena*, *Querula*) [2,3], or Asian broadbills (e.g., *Cymbirhynchus*, *Eurylaimus*) [4].

The discovery of yet another new carotenoid in the feathers from *Oriolus* implies that there could be additional undetermined molecular diversity in avian carotenoid physiology and pigmentation. Further investigation of exotic carotenoid plumages is warranted and ongoing.

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