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Novel methoxy-carotenoids from the burgundy-colored plumage of the Pompadour Cotinga *Xipholena punicea*

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ABSTRACT

Recent advances in the fields of chromatography, mass spectrometry, and chemical analysis have greatly improved the efficiency with which carotenoids can be extracted and analyzed from avian plumage. Prior to these technological developments, Brush (1968) [1] concluded that the burgundy-colored plumage of the male pompadour Cotinga *Xipholena punicea* is produced by a combination of blue structural color and red carotenoids, including astaxanthin, canthaxanthin, isozeaxanthin, and a fourth unidentified, polar carotenoid. However, *X. punicea* does not in fact exhibit any structural coloration. This work aims to elucidate the carotenoid pigments of the burgundy color of *X. punicea* plumage using advanced analytical methodology. Feathers were collected from two burgundy male specimens and from a third aberrant orange-colored specimen. Pigments were extracted using a previously published technique (McGraw et al. (2005) [2]), separated by high-performance liquid chromatography (HPLC), and analyzed by UV/Vis absorption spectroscopy, chemical analysis, mass spectrometry, nuclear magnetic resonance (NMR), and comparison with direct synthetic products. Our investigation revealed the presence of eight ketocarotenoids, including astaxanthin and canthaxanthin as reported previously by Brush (1968) [1]. Six of the ketocarotenoids contained methoxyl groups, which is rare for naturally-occurring carotenoids and a novel finding in birds. Interestingly, the carotenoid composition was the same in both the burgundy and orange feathers, indicating that feather coloration in *X. punicea* is determined not only by the presence of carotenoids, but also by interactions between the bound carotenoid pigments and their protein environment in the barb rami and barbules. This paper presents the first evidence of metabolically-derived methoxy-carotenoids in birds.

Introduction

Carotenoid pigments produce the yellow, orange, red, and pink coloration of many avian feathers, skin, scales and ramphothecae. To date, 24 different carotenoids have been identified in the plumages of over 150 species of birds [3]. Among all birds, the unusual purple, violet, or burgundy plumage coloration of several Neotropical cotingas (Cotingidae) have received specific attention, especially the deep purple breast and throat feathers of the males in the genus *Cotinga*, and the deep crimson colors of males of the genus *Xipholena* (Fig. 1) which are produced without the involvement of melanin. Görnitz and Rensch [4] named the unusual burgundy and violet pigments they extracted from feathers from these genera "cotingin." By examining spectral absorption, Völker [5] tentatively identified *Xipholena* and *Cotinga* pigments as having similar spectral characteristics to the pigments of some ibis and flamingos, which have since been reported to be comprised primarily of canthaxanthin (Fig. S1) [1]. However, Völker also described unusual heat and pressure sensitive properties of these *Cotinga* feathers, and suggested that their unusual color might be a collaboration between pigmentary absorbance and novel feather structure. He further suggested that the name cotingin be abandoned. Most recently, Brush [1] conducted a thin layer chromatographic (TLC)[1] analysis of solvent-extracted pigments from the feathers of the Pompadour Cotinga, *Xipholena punicea* (hereafter

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[1] *Abbreviations used:* HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin layer chromatographic; MTBE, methyl-tert butyl ether; MS, mass spectra.

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Fig. 1. A male Pompadour Cotinga, *Xipholena punicea* (Cotingidae) from Les Nourages, French Guiana. (Photo by Tanguy Deville, published with permission).

X. punicea) (Fig. 1), and identified its components as the carotenoids astaxanthin, canthaxanthin, isozeaxanthin, along with a fourth unidentified highly polar carotenoid.

Perhaps because two of these four carotenoid pigments were well known from other bird plumages that lack the distinct purplish hue, Brush [1] concluded that the violet color was due to a combination of ketocarotenoid pigments and a blue structural color. Brush's [1] claim has been repeated recently in a review of carotenoid plumage coloration [3]. However, there are two clear problems with the combined structural-pigmentary hypothesis. In the genus *Cotinga*, blue structural coloration is produced by constructive interference (or coherent scattering) of light waves scattered by nanoscale air bubbles in the medullary cells of the feather barb rami [6,7]. The genus *Xipholena*, however, exhibits no blue or green structural coloration at all. Furthermore, *Xipholena* is phylogenetically related to three other genera that entirely lack blue feather structural coloration: *Carpodectes*, *Conioptilon*, and *Gymnoderus* [8,9]. In addition, by examining feathers of *Xipholena* and *Cotinga* species with a dissection microscope, it is readily observed that the burgundy or purple hues are uniformly present in both barb rami and barbules (Fig. S2). Furthermore, the color remains virtually identical in reflected or transmitted light (Fig. S2), which falsifies the hypothesis of any role for medullary barb structural color in the production of this color. Thus, we can reject Brush's hypothesis. With advent of improved procedures for the extraction of pigments from bird feathers, and advances in chromatographic and mass spectrometry methods since Brush [1], we initiated a new examination of the identity and coloration mechanism of the carotenoids of *X. punicea*.

Materials and methods

Extraction of the carotenoids from feathers

Feathers were obtained from two male *X. punicea* from the Yale Peabody Museum of Natural History: a 19th century specimen from British Guiana (YPM 1133) and a new specimen received in 2009 from the Dallas World Aquarium and Zoo from Suriname (YPM 84426). Additionally, feathers from an aberrant, orange specimen of *X. punicea* (AMNH 494642) were obtained from the American Museum of Natural History (Fig. S3). The results from all three specimens were very similar. Feather pigments were extracted using a previously published method [2,10] with minor modifications. Briefly, 10–12 feathers were soaked in ethanol (J.T. Baker, technical grade) followed by hexanes (J.T. Baker, technical grade) in order to remove surface lipids. Pigmented barbs were then detached using a razor and placed into a 10 mL glass test tube. The barbs were covered with acidified pyridine prepared by the addition of 4 drops of hydrochloric acid (J.T. Baker) to 100 mL pyridine (J.T. Baker) [11]. After flushing with argon gas, the tube was capped and placed in a 95 °C water bath. The tube was removed from the bath after ~30 min when the loss of pigmentation from the barbs appeared complete. The extract was then allowed to cool to on ice, after which time the carotenoid pigments were separated from dissolved lipids by the addition of a 3:1 (v/v) solution of methyl-tert butyl ether (MTBE, Fisher Scientific) and water (Sigma–Aldrich) [12]. Carotenoid pigments transferred readily into the ether phase while the lipids remained the aqueous pyridine phase giving it a cloudy appearance. The pigmented ether phase was then partitioned with additional water until the aqueous solution was clear indicating few lipids remained, at which time the ether layer was collected, dried under nitrogen gas, and stored at −80 °C for subsequent analysis.

In order to assess whether the extraction protocol resulted in chemical modification of the carotenoids, control standards of adonirubin (BASF), astaxanthin (Roche), canthaxanthin (Roche), isozeaxanthin (BASF), lutein (Douglas Laboratories), and zeaxanthin (Roche) (Fig. S1) were subjected to the same procedure described above and then analyzed using the HPLC methods described below. While the procedure was found to result in a portion of the carotenoids being isomerized from all-trans to cis configurations, due to thermalization in the hot water bath, essentially no chemical modification of the carotenoids was observed. Moreover, a large portion of the cis-isomers that were formed reconverted into trans isomers by leaving the samples at room temperature in the dark overnight. Lastly, to be absolutely certain that the newly discovered pigments were not artifacts of the extraction protocol, several feathers were ground mechanically for 15 min using a mortar and pestle in the presence of acetone, and the resulting extract was filtered, dried under nitrogen gas, injected into the HPLC, and analyzed using the protocol described below. The resulting chromatogram was very similar to that seen from the sample obtained using acidified pyridine. Eight major pigments were found, and only minor variations in relative peak heights were observed.

High-performance liquid chromatography

Dried pigment extract obtained from the feather barbs was dissolved in hexanes/acetone, 86:14 (v/v) (Fisher Scientific) and filtered through a nylon syringe filter (Grace Davison Discovery Sciences, 4 mm, 0.20 μm). The sample was then injected into a Waters 600E HPLC system employing a normal-phase Phenomenex Luna 5μ silica column (250 × 4.6 mm) and equipped with a Waters 2996 photodiode array detector. The chromatographic protocol was programmed to run at a flow rate of 1.5 mL/min for 60 min using a linear gradient from 90:10 to 80:20 hexanes/acetone (v/v) [13]. Individual peaks eluting from the HPLC were collected, dried, and re-dissolved in methanol for absorption spectroscopy.

Purified adonirubin, adonixanthin (BASF), astaxanthin, canthaxanthin, echinenone (BASF), isozeaxanthin, lutein, rhodoxanthin (Carotenature), violerythrin and zeaxanthin, as well as α-doradexanthin (Fig. S1) isolated from *Cardinalis cardinalis* feathers [14,15], were run as controls using the normal-phase HPLC protocol for direct comparison of the retention times with those from the pigments isolated from the *X. punicea* feathers.

The protocol used for HPLC analysis was adopted after an extensive investigation of many alternatives. Earlier attempts at obtaining purified pigments via HPLC yielded poorly resolved

chromatograms due to column modifications induced by the presence of lipids and other impurities, which were confirmed by mass spectral analysis. Partitioning the pigment extract several times with MTBE as described by McGraw et al. [12] prior to carrying out the HPLC analysis resulted in a significant improvement in the reproducibility and resolution of the chromatograms and in the elimination of extraneous peaks from the mass spectra.

A Waters NovaPak RP-C18 reversed-phase HPLC column and protocol was used to further purify pigments that co-eluted using the normal-phase column. The mobile phase had a flow rate of 1.0 mL/min and began with a 15 min isocratic delivery of a solvent mixture that was 99% acetonitrile/methanol/water, 87:10:3 (v/v/v) (acetonitrile and methanol, Fisher Scientific, HPLC grade; water, Sigma, HPLC grade) and 1% ethyl acetate (Fisher Scientific, HPLC grade). From 15 to 40 min, the solvent was adjusted by linear gradient to 60% acetonitrile/methanol/water, 87:10:3 (v/v/v) and 40% ethyl acetate. The injection solvent in the reversed-phase HPLC protocol was acetonitrile.

Mass spectrometry

The molecular weights of HPLC purified pigments were determined using a Fisons VG Quattro II mass spectrometer (APCI+ and APCI− modes) controlled by MassLynx software. Prior to the run, the pigments were dissolved in MTBE/methanol, 50:50 (v/v) (Fisher Scientific) and injected under the following conditions: cone voltage, 15 V; probe temperature, 480 °C; and source temperature, 110 °C. The parent ion peaks observed using this instrument were further confirmed by running the same samples on an Applied Biosystems QTRAP 4000 controlled by Applied Biosystems Analyst software. On this machine, samples were dissolved in MTBE/methanol, 50:50 (v/v), methanol, or acetonitrile depending on solubility, and injected using an infusion pump with a delivery rate of 50 μL/min. The instrument parameters were as follows: 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 for APCI+ and −130 V for APCI−; collision energy, 10 eV for MS and 35 eV for MS–MS. An HPLC/quadrupole time-of-flight system (Alliance 2695 HPLC, Waters Corp., Milford, MA; QTof Premier, Micromass UK, Manchester, UK) was used to determine accurate masses for HPLC peaks 6 and 8 (see below), each dissolved in 50:50 (v/v) MTBE/methanol. A mobile phase of 0.2% ammonium acetate/methanol/MTBE was applied as a linear gradient from 10/90/0 to 0/90/10 (v/v/v) over 10 min then to 7/60/33 over 2 min followed by re-equilibration for 2 min at a flow rate of 1.8 mL/min. The HPLC eluent was interfaced with the QTof via an atmospheric pressure chemical ionization probe operated in both positive and negative polarity (separate HPLC runs). Mass spectra were acquired in V-mode (~8000 resolution) from 100–1000 m/z with peaks centroided, a scan time of 0.3 s and dynamic range enhancement enabled. At intervals of 30 s, a 0.1 s lockspray scan was acquired with leucine enkephalin as the lockspray compound (554.2615 m/z) to correct for minor deviations in calibration. Prior to analysis, the QTof was fully calibrated in negative mode from 114 to 1473 m/z and from 91 to 1451 m/z in positive mode using sodium formate solution. The resultant MS spectra were analyzed with MassLynx software, V4.1 (Micromass UK, Manchester, UK) and i-FIT, an isotope pattern scoring algorithm within MassLynx. These tools score the observed monoisotope accurate mass and isotope pattern versus theoretical values based on empirical formulae. Source parameters included: 30 μA corona current, 500 °C probe, 110 °C source block, 35 V cone, 50 L/h cone gas (N_2), 400 L/h desolvation gas (N_2), collision energy 8 eV with argon CID gas (4.2×10^{-3} mbar). A different QTof Premier (Micromass UK, Manchester, UK) instrument without an HPLC front-end was used to determine accurate masses for HPLC peaks 4 and 7 (see below) using the same conditions as described above.

Nuclear magnetic resonance spectroscopy

^{1}H-NMR spectroscopy was carried out on three of the six novel carotenoid peaks isolated by HPLC using either a Bruker Avance 500 MHz or a Varian Inova 600 MHz spectrometer on samples dissolved in either $CDCl_3$ or CD_3OD (Cambridge Isotope Laboratories Inc., USA). TMS was used as an internal standard.

Chemical reactivity and analysis

Reaction with sodium borohydride

Absorption spectroscopy was used to monitor the reaction of the HPLC-purified carotenoids with sodium borohydride to determine the presence of conjugated carbonyl groups. The procedure used approximately 100 μg of sodium borohydride (Acros Organics) added to the carotenoid dissolved in ~2 mL of methanol and having an optical density at its λ_{max} of 0.05–0.10 in a 1 cm path length quartz cuvette [16,17]. Absorption spectra were taken at 5 min intervals to monitor the progress of the reaction through to completion, which typically took 20 min.

Reaction with acetic anhydride

Acetic anhydride slowly converts hydroxyl groups to acetyl groups and reduces the polarity of the molecule without any significant affect on its absorption spectrum. The number of products observed in an HPLC chromatogram of the reaction mixture as a function of time will reveal the number of hydroxyl groups. For example, a carotenoid with a symmetrical placement of two hydroxyl groups on opposite rings will produce mono- and di-acetylated products at roughly evenly spaced retention time intervals, whereas a carotenoid with two hydroxyl groups either on the same ring or asymmetrically placed on two opposite rings may produce two different mono-acetylated intermediates which will elute with similar retention times in the HPLC, in addition to a fully acetylated product [17]. Standards of adonirubin, astaxanthin, canthaxanthin, lutein and zeaxanthin were run as controls of molecules having different numbers and/or positions of hydroxyl groups. Aliquots of the reaction mixture were taken at the beginning of the reaction, two hours after the reaction began, and after the reaction had run to completion overnight to assess the composition of the reaction mixture and to deduce the number and symmetry of the attached hydroxyl groups. The aliquots were then analyzed by HPLC. Zeaxanthin was acetylated as a control alongside each of the purified HPLC peaks to confirm the integrity of the reagents.

The number of hydroxyl groups in the carotenoids was determined by reacting the extracted pigments with acetic anhydride and monitoring the products using HPLC. The acetylation method previously described in literature [17] was modified slightly, whereby the individual pigments were dissolved in 3 mL of pyridine (dried with molecular sieves, Sigma–Aldrich) to an absorbance of 0.1–1 in a 1 cm path length cuvette. One mL aliquots of each sample were then placed into three individual amber glass vials at room temperature in the dark. Fifty microliters of acetic anhydride was added to two of the vials, while the third vial served as a control. The control vial and one treated vial were allowed to incubate for 2 h in the dark at room temperature, while the third vial remained in incubation overnight. At the end of the incubation times, ~4 mL of MTBE/water, 1:1 (v/v) were added to the reaction mixtures, and they were subsequently washed four times with water. The MTBE phase containing the carotenoids was dried using a gentle stream of nitrogen gas and then injected into the HPLC employing either the normal-phase or reversed-phase protocol described above, depending on the polarity of the original molecule.

A bona fide zeaxanthin standard was reacted with the acetic anhydride in parallel with each component of the feather extract to serve as an additional control.

Saponification

A previously described saponification method was used to test for ester linkages in the carotenoids [17]. Following this procedure the total feather pigment extract was dissolved in 5 mL of diethyl ether, to which 5 mL of 10% methanolic KOH was added. The mixture was then left overnight in the dark at room temperature, after which time the treated carotenoids were extracted using additional diethyl ether and washed with distilled water several times to remove the base. The diethyl ether solution containing the carotenoids was then dried under nitrogen gas and subjected to the normal-phase HPLC analysis described above. Only minor changes in the relative intensities of the peaks in the normal-phase HPLC chromatogram were observed after saponification indicating none of the carotenoids extracted from the feathers contained ester linkages.

Plumage reflectance spectrum modeling

The reflectance spectrum of normal male (YPM 1133) and the aberrant male (AMNH 494642) *X. punicea* were measured using standard protocols [18]. A predictive model of the reflectance spectrum of the extracted carotenoid pigments was produced from a weighted average of the absorbance of the component extracted carotenoids following: % Reflectance = $100(10^{-A})$ where A is the absorbance of each carotenoid. The relative contribution of each pigment was computed from the integrated area of the HPLC peaks corrected for variation in both the absorbance at the HPLC detection wavelength and the molar absorption coefficients of the pigments which were calculated from their $A^{1\%}$ values using the molecular weights determined from mass spectrometry [19]. The mass percent of each carotenoid is given in Table 1. Measured and predicted reflectance spectra were compared with peak reflectance normalized to 1.

Results

Chemical and spectral analysis

The carotenoids obtained from the feathers of both the standard and aberrant male *X. punicea* specimens produced eight major HPLC peaks and several minor ones resolved with baseline separation (Fig. 2). The fact that the two burgundy specimens – one century old wild specimen and one contemporary zoo bird – contained virtually identical pigment compositions and were very similar to that of the orange aberrant specimen (Figs. 2 and S2) suggests that the differences in color are not due to differences in pigment composition.

Bona fide standards of adonirubin, adonixanthin, astaxanthin, canthaxanthin, α-doradexanthin, echinenone, isozeaxanthin, lutein, rhodoxanthin, violerthryin and zeaxanthin (Fig. S1) were analyzed to calibrate the retention times in the HPLC protocol. Comparing the retention times of these standards with those observed from each of the peaks from the feather extracts provided insight into the structure of the *Xipholena* carotenoids. For example, zeaxanthin which has hydroxyl groups at the 3- and 3′-positions, was found to have a longer retention time by ~7 min compared to isozeaxanthin which has hydroxyl groups at the 4- and 4′-positions. Thus, a carotenoid possessing an hydroxyl group at the 3- or 3′-position can be considered more polar than one having an hydroxyl group at the 4- or 4′-position. In contrast,

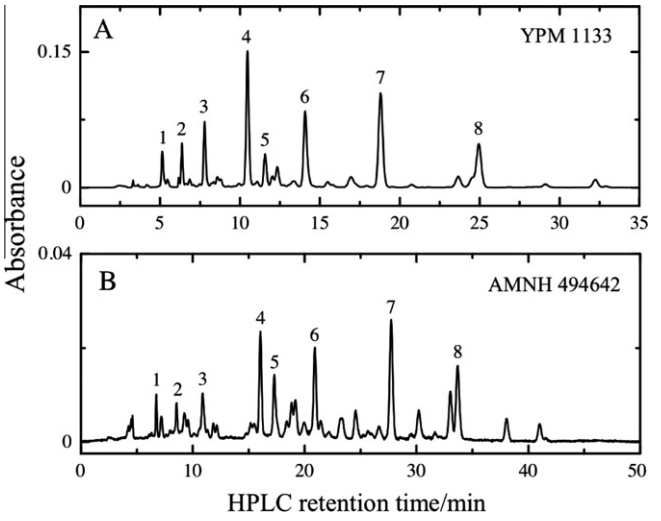

Fig. 2. Normal-phase HPLC chromatogram of the total carotenoid extract from (A) burgundy-colored *X. punicea* feathers (YPM 1133) and (B) orange-colored feathers from the aberrant specimen (AMNH 494642). The chromatogram was detected at 470 nm. In both cases, carotenoids were extracted as described in the text, and then left in the dark at room temperature overnight prior to the chromatographic procedure. This allowed unstable cis-isomers to reconvert into all-trans configurations.

Table 1
HPLC retention times and parent ion mass values obtained from a mass spectrometry analysis of the carotenoids isolated from *X. punicea* feathers. The structures were assigned on the basis of these data and other characteristics as described in the text.

HPLC peak	Assigned carotenoid (alternate name)	Proposed chemical name	HPLC retention time, t_R (min)	Mass percent of total carotenoids	Parent ion mass (m/z)
1	Canthaxanthin	–	5.1	7.4	564
2	3-Methoxy-β,β-carotene-4,4′-dione(3-methoxy-canthaxanthin)	Brittonxanthin	6.3	8.2	594
3	3,3′-Dimethoxy-β,β-carotene-4,4′-dione(3,3′-dimethoxy-canthaxanthin)	pompadourin	7.7	11.1	624
4	3-Methoxy-3′-hydroxy-β,ε-carotene-4-one	Xipholenin	10.5	28.4	596.4175[a]
5	Astaxanthin	–	11.5	6.1	596
6	3,3′-Dimethoxy-2,3-didehydro-β,β-carotene-4,4′-dione	2,3-Didehydro-pompadourin	14.0	13	622.4018
7	3′-Hydroxy-3-methoxy-2,3-didehydro-β,ε-carotene-4-one	2,3-Didehydro-xipholenin	18.8	18.4	594.4064[a]
8	3,3′-Dimethoxy-2,3,2′,3′-tetradehydro-β,β-carotene-4,4′-dione	Cotingin	24.9	7.4	620.3865

[a] For these peaks, (M + Na)+ appeared as the dominant ion. Therefore, the mass given here represents this value minus the mass of sodium (22.9897 m/z).

carbonyls at the 4- and 4′-positions do not give rise to a very polar molecule in this series, as evidenced by the fact that canthaxanthin has a relatively short retention time of ~5 min. Similar deductions could be made from running standards with different varieties of functional groups (Fig. S1).

It was not uncommon to observe minor differences in the HPLC retention times in different chromatographic runs of the standards compared to the carotenoids from the feather extract. To resolve this issue, purified control molecules were directly incorporated ("spiked") into aliquots of the extract to serve as internal standards. This was done using adonirubin, adonixanthin, β-carotene, α-doradexanthin, echinenone, isozeaxanthin, lutein and zeaxanthin. These results were then used to determine the relative polarity, and therefore the potential structural characteristics, of pigments from the *X. punicea* extracts.

Absorption spectra recorded on the HPLC diode array and also on the UV/Vis spectrophotometer were used to deduce structural characteristics of the molecules (Fig. 3). The λ_{max} of the absorption spectral bands indicates the π-electron chain length and chromophore identity, because carotenoids having longer systems of linearly conjugated double bonds will absorb at longer wavelengths [20,21]. HPLC peaks 6 and 8 were red-shifted relative to the other peaks indicating longer conjugated chain lengths for these molecules compared to the others. The spectra for all eight of the major peaks were broad and lacked well-resolved vibronic features, (i.e. multimodal peaks in the absorbance spectrum) typical of carbonyl-containing xanthophylls [20,21]. Peaks 4 and 7 displayed a small amount of vibronic structure similar to that of adonixanthin, suggesting only one carbonyl in conjugation. All other peaks displayed very broad spectra similar to that of canthaxanthin, which has two carbonyl groups in conjugation. Upon trans-to-cis isomerization the main absorption bands of carotenoids will shift 2–6 nm to shorter wavelength, and a small peak ~140 nm to shorter wavelength from the main band will appear [21]. This is observed for several of the minor HPLC peaks in the chromatogram. Thus, based on absorption spectra and proximity of retention time to their all-trans counterparts, these are believed to be cis-isomers formed during the extraction procedure, which involved heating the feather barbs to 95° C to release the pigments. Also, many of these minor peaks disappeared after several hours at room temperature, further supporting their assignment to unstable cis-isomers that revert spontaneously to the more thermodynamically stable all-trans configuration.

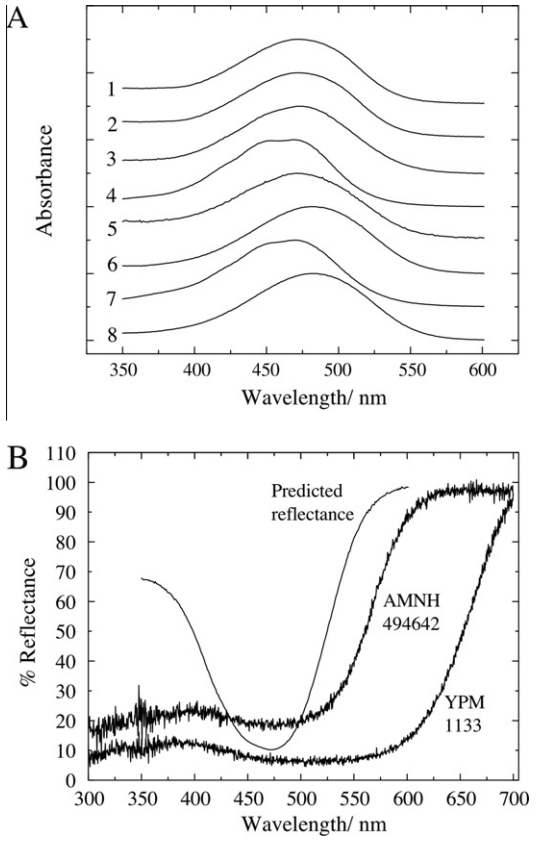

Fig. 3. (A) Absorption spectra of the carotenoids associated with the HPLC peaks 1–8 shown in Fig. 2A. The spectra were taken in methanol at room temperature, normalized to their λmax values, and offset for clarity of presentation. (B) Normalized reflectance spectra of normal, burgundy-colored male *X. punicea* feathers (YPM 1133), and aberrant orange male feathers (AMNH 494642), and a predicted reflectance spectrum based on the absorption spectra and relative abundance of the eight carotenoid pigments extracted from *X. punicea* . The burgundy feather reflectance shows a substantial bathochromic shift of ~93 nm, where as the aberrant orange feathers closely matches the reflectance prediction based on the pigments in solution.

Sodium borohydride reduction of a carbonyl in conjugation with the π-electron chain of the carotenoid to an hydroxyl group decreases the extent of π-electron delocalization, blue-shifts the main absorption band and sharpens the vibronic structure in the spectrum, thus revealing the number of carbonyl groups and helping to determine the basic structure of the chromophore [17]. Based on a comparison with the behavior of the purified standards, adonixanthin, astaxanthin, canthaxanthin and echinenone (Fig. S1), a blue-shift of approximately 25 nm indicates the presence of at least two carbonyl groups in conjugation, whereas a shift of approximately 15 nm indicates the presence of only one carbonyl group in conjugation with the system of π-electron carbon–carbon double bonds. As expected from the broad absorption spectral lineshapes (Fig. 3), all of the molecules were affected by the addition of sodium borohydride, suggesting the presence of at least one conjugated carbonyl. Upon chemical reduction, HPLC peaks 1, 2, 3, 5, 6 and 8 showed a red-shift of ~25 nm indicating the presence of at least two carbonyls, while peaks 4 and 7 showed red-shift of ~15 nm, indicating the presence of only one conjugated carbonyl. The reduction products of peaks 4 and 7 were purified by HPLC and analyzed by mass spectrometry, which revealed that the reduction products had a net gain of 2 mass units each, indicating that only one carbonyl had been reduced. Peaks 6 and 8 were unstable upon treatment with sodium borohydride, precluding further analysis via mass spectrometry. Using this chemical reduction method, it was found that the carotenoids associated with HPLC peaks 4 and 7 have an α-carotene-like chromophore containing one β-ring and one ε-ring. All other HPLC peaks yield β-carotene-like chromophores with two β-rings.

The acetylation reaction and analysis revealed that, of the six unknown pigments associated with HPLC peaks 2, 3, 4, 6, 7 and 8, only the carotenoids associated with HPLC peaks 4 and 7 showed the presence of a single hydroxyl group.

Mass spectral analysis revealed the molecular weights of all of the HPLC peaks. The results are summarized in Table 1, and illustrated by the data for peaks 1–3 given in Fig. 4. The molecular weights found for these carotenoids are significantly higher than the 536.88 *m/z* value for the parent α- and β-carotene hydrocarbons, suggesting the presence of multiple functional groups containing oxygen.

Pigment identification

Brush [1] reported that the feathers of *X. punicea* contained four primary carotenoids, astaxanthin, canthaxanthin, isozeaxanthin, and a fourth unidentified highly polar carotenoid. It is clear from the well-resolved HPLC chromatogram shown in Fig. 2 that Brush was correct about the presence of an highly polar carotenoid, but

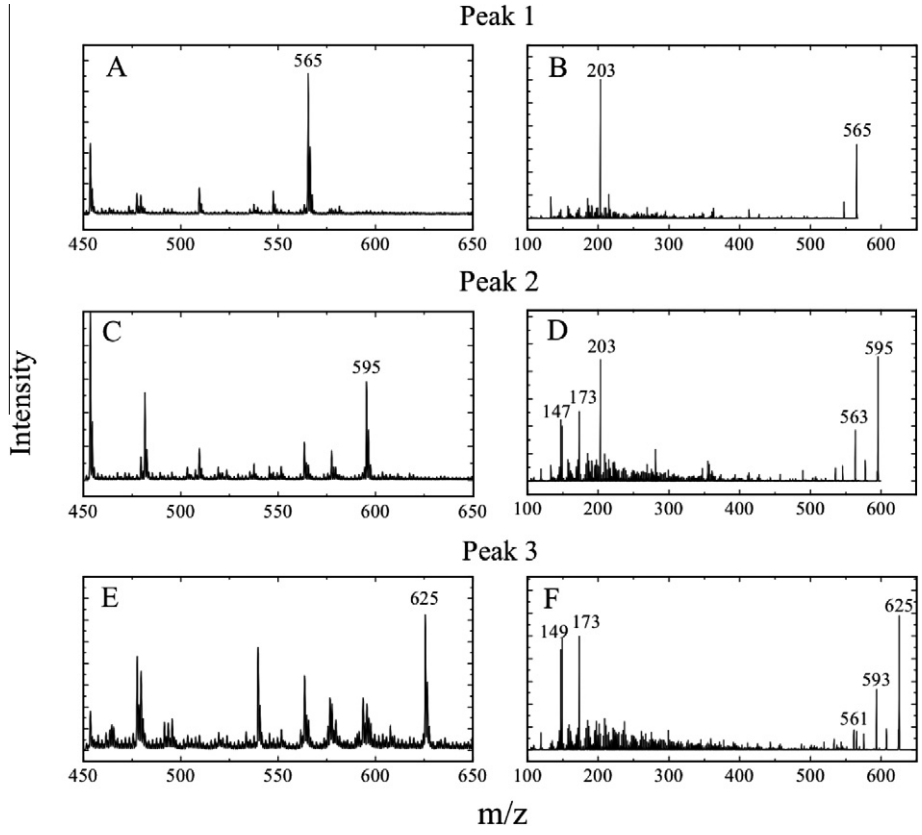

Fig. 4. Mass spectra (MS) and MS–MS traces of peaks 1 (A and B), 2 (C and D), and 3 (E and F) obtained using the QTRAP 4000 instrument as described in the text.

the composition of these feathers is much more complex than originally thought. The present work revealed the *X. punicea* feather extracts to have eight major carotenoids, all of which are keto-derivatized α- or β-carotene structures, and some of which possess other functional groups containing oxygen that derive from the ability of this species, and birds in general, to metabolize common avian dietary carotenoids through dehydrogenation or oxygenation reactions [1,15,22].

Peak 1 is assigned to canthaxanthin (β,β-carotene-4,4′-dione) based on the observations that its retention time and absorption spectrum (λ_{max}, 470 nm in methanol) match that of a bona fide standard. This identification was further supported by the results of the sodium borohydride reduction and mass spectral analysis, which revealed a parent ion peak at 564 *m*/*z* (mass-to-charge ratio) and a 203 *m*/*z* fragment, which correspond to the molecular weight and 4-keto β-end group ($C_{14}H_{19}O$) respectively of canthaxanthin.

Peak 2 was found to be slightly more polar than canthaxanthin and displayed a similar broad absorption spectrum with a λ_{max} of 471 nm in methanol, although with a slight vibronic inflection and asymmetry on the short wavelength edge of the spectrum. This could derive from a small decrease in conformational disorder brought about by functional group substitution on one or more of the rings. As observed with peak 1, chemical analyses indicated the presence of 2 carbonyls and no hydroxyl groups. Mass spectral analysis revealed a parent ion peak at 594 *m*/*z*, as well as a mass fragment in the MS-MS trace at M-32 *m*/*z* (Fig. 4D) indicating the loss of a methoxyl functional group plus an α-hydrogen. Furthermore, a 203 *m*/*z* ion peak representing the 4-keto β-end group of canthaxanthin was observed (Fig. 4B and D). Based on these data peak 2 is assigned to 3-methoxy-β,β-carotene-4,4′-dione (a.k.a. 3-methoxy-canthaxathin, Fig. 5). It should be pointed out that these data cannot unambiguously assign the position of the methoxyl group. However, oxidation reactions of carotenoids tend to activate the 3- and 3′-carbon positions which are alpha to the carbonyl. (See Discussion below.)

Peak 3 showed an identical absorption spectrum to peak 2, and the same behavior when treated with sodium borohydride as was observed for peaks 1 and 2. Treatment with acetic anhydride indicated the absence of hydroxyl groups. The molecular weight was determined by mass spectrometry to be 624 *m*/*z*, with fragment ions at M-32 *m*/*z* and M-64 *m*/*z*, suggesting the loss of two methoxyl groups. Furthermore, the 203 *m*/*z* ion fragment seen for peaks 1 and 2 was absent, indicating functionalization of both β-rings. Based on these data, and following from the identifications of peaks 1 and 2, this peak is assigned to 3,3′-dimethoxy-β,β-carotene-4,4′-dione (a.k.a. 3,3′-dimethoxy-canthaxanthin, Fig. 5). In order to verify this assignment, we synthesized this molecule using a procedure previously described by Bhosale et al. [23] for producing 3-methoxy-zeaxanthin from zeaxanthin. Briefly, 10 mg of astaxanthin was dissolved in 1 mL of dimethylsulfoxide, and 0.75 mL dimethylformamide, 0.5 mL of methyl iodide and 100 mg of barium oxide were added. The reaction mixture was stirred at 35 °C for 48 h, extracted with chloroform and washed several times with water. The organic layer was dried over anhydrous sodium sulfate and concentrated. The resulting crude product was injected into the HPLC running the normal-phase protocol described above. Pure 3,3′-dimethoxy-β,β-carotene-4,4′-dione was obtained and characterized by absorption spectroscopy and mass spectrometry. The molecular weight of the synthetic molecule and its HPLC retention time were found to be identical to that of peak 3 confirming this component of the *X. punicea* feather extracts to be 3,3′-dimethoxy-β,β-carotene-4,4′-dione or 3,3′-dimethoxy-canthaxathin (Fig. 5).

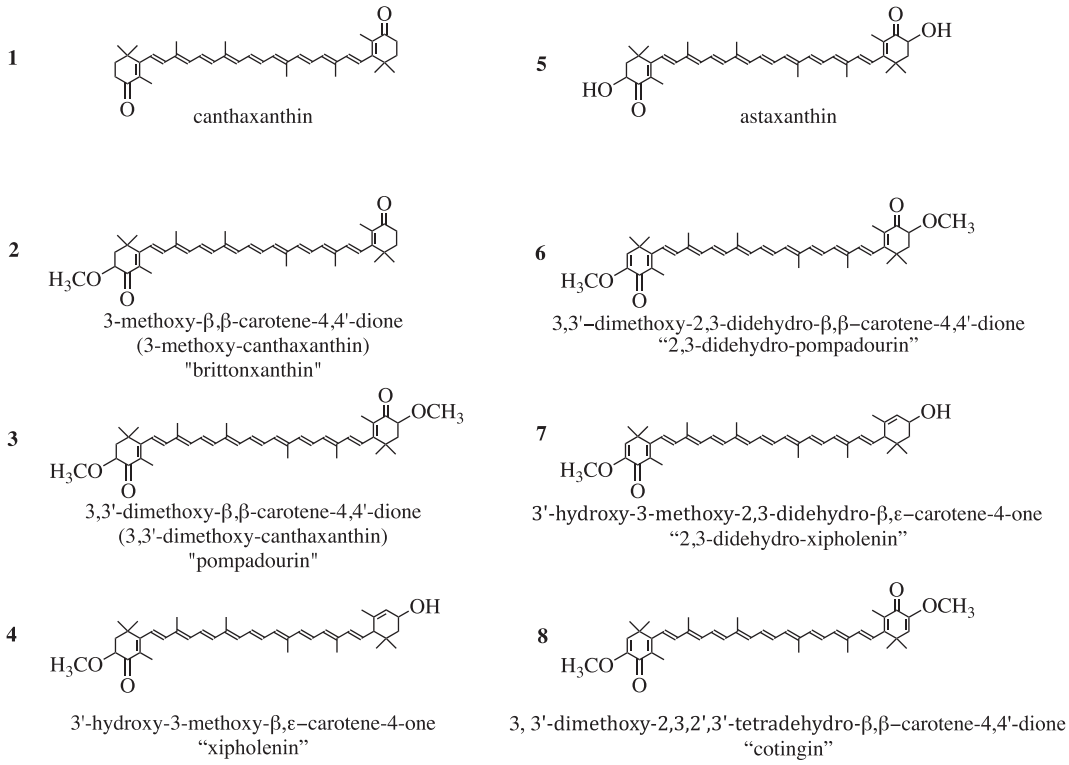

Fig. 5. Carotenoid structures assigned to HPLC peaks 1–8 (Fig. 2).

HPLC peak 4 displayed a broad absorption spectrum with very slight vibronic structure and a λ_{max} at 461 nm in methanol. The spectral lineshape of peak 4 is more similar to that of an adonixanthin standard, which has only one carbonyl in conjugation. Sodium borohydride reduction confirmed the presence of only one conjugated carbonyl and an α-carotene chromophore. Acetic anhydride treatment confirmed the presence of a single hydroxyl group. Mass spectral analysis displayed a molecular ion at 596 *m*/*z*, the loss of an hydroxyl group was indicated by M-18 *m*/*z*, and the loss of a methoxyl group indicated by M-32 *m*/*z*. High-resolution mass spectrometry of peak 4 confirmed a molecular ion at 619.4072, indicative of a mass of 596.4175 plus a sodium adduct. This mass is consistent with the empirical formula $C_{41}H_{56}O_3$ with a mass accuracy of 8.2 ppm. The presence of a single methoxyl group with a resonance at 3.5 ppm was confirmed by proton NMR (Fig. 6B). On the basis of these data, peak 4 is proposed to be 3′-hydroxy-3-methoxy-β,ε-carotene-4-one (Fig. 5). Other arrangements of the attached functional groups cannot be ruled out, however, the metabolic pathway that has produced the methoxylated β-rings of peaks 2 and 3 seems likely to produce the same derivative in peak 4.

Also in agreement with previous work by Brush [1], peak 5 is identified as astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) based on co-chromatography with a bona fide standard. Furthermore, the absorption spectrum (λ_{max}, 468 nm in methanol), sodium borohydride reduction product (22 nm blue-shift), and mass spectrometry (parent ion, 596 *m*/*z*) are all consistent with data obtained from an astaxanthin standard.

Peak 6 has a broad absorption spectrum with a λ_{max} red-shifted to 478 nm in methanol indicating a longer conjugated π-electron chain length than the previously analyzed peaks. This is highly suggestive of the π-electron conjugation being extended into at least one of the β-rings containing an additional double C=C bond. Chemical tests indicated at least two carbonyls in conjugation, and ruled out the presence of hydroxyl groups. High-resolution

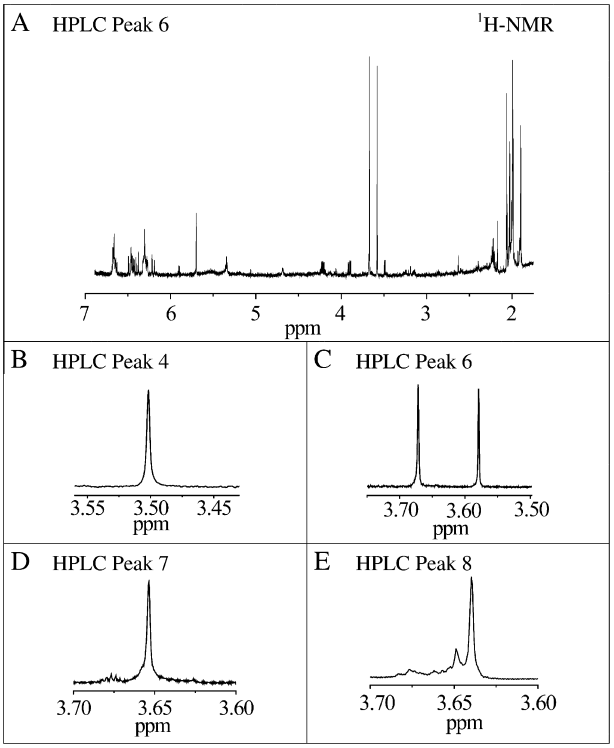

Fig. 6. 1H-NMR spectra from (A) HPLC peak 6, and (B) HPLC peak 4, (C) HPLC peak 6, and (D) HPLC peak 7 in the 3.4–3.7 ppm region where methoxyl groups are evident. Alkene protons are seen in (A) above 6.0 ppm. Aliphatic methyl groups are observed around 2 ppm. HPLC peak 4 (B) shows a single methoxyl group at 3.5 ppm. HPLC peak 6 (C) shows two non-equivalent methoxyl groups at 3.58 and 3.66 ppm. HPLC peak 7 (D) shows one methoxyl group at ~3.65 ppm. HPLC peak 8 (E) shows one methoxyl group at ~3.64 ppm.

mass spectral analysis revealed a molecular mass at 622.4018 *m*/*z* which yielded an empirical formula of $C_{42}H_{54}O_4$ with a mass accuracy of 0.6 ppm. A fragmentation ion at M-32 *m*/*z* indicated the presence of at least one methoxyl group. NMR spectroscopy revealed two separate methoxyl resonances (Fig. 6A and C), consistent with two methoxyl groups being present on magnetically non-equivalent terminal rings. Thus, we propose that one of the terminal β-rings of this carotenoid contains a double bond between carbons 2 and 3, and assign the molecule as 3,3′-dimethoxy-2,3-didehydro-β,β-carotene-4,4′-dione (Fig. 5).

Peak 7 has a very broad absorption spectrum, a λ_{max} of 462 nm in methanol, and the slight vibronic fine structure of a molecule containing only one carbonyl in the conjugated chain. Sodium borohydride reduction of this peak indicated the presence of only one conjugated carbonyl and an α-carotene-like chromophore. Mass spectral analysis of the sodium borohydride reduction product confirmed the addition of only two mass units, consistent with only one carbonyl being present. Like HPLC peak 4, the reduction product of peak 7 has the absorption spectrum of an α-carotene chromophore. Mass spectral analysis revealed a molecular mass of 594 *m*/*z*, with the loss of M-32 *m*/*z* apparent in the MS-MS spectrum. The presence of one methoxyl group was confirmed by NMR (Fig. 6C). High-resolution mass spectral analysis displayed a major ion at 617.3961 *m*/*z* which represents (M + Na)+. When the mass of the sodium adduct is deducted, this measurement yielded a mass of 594.4064 *m*/*z* with a mass accuracy of 0.5 ppm. This is consistent with the empirical formula $C_{41}H_{54}O_3$. On the basis of these data, and analogous to the structure of peak 6, we propose peak 7 to be 3′-hydroxy-3-methoxy-2,3-didehydro-β,ε-carotene-4-one (Fig. 5).

Peak 8 co-elutes with two other carotenoids and one cis-isomer, and therefore was further purified using the reversed-phase HPLC method described above. The resulting dominant pigment displayed a broad absorption spectrum with a relatively long λ_{max} at 482 nm in methanol which, like peak 6, is suggestive of the extension of the π-electron conjugation into both of the β-rings. Despite the apparent very polar nature of this peak, acetic anhydride treatment did not yield a positive test for hydroxyl groups. High-resolution mass spectral analysis revealed a molecular ion of 620.3865 *m*/*z* consistent with an empirical formula of $C_{42}H_{52}O_4$ (0.2 ppm mass accuracy); i.e. two mass units less than that of peak 6. MS-MS fragmentation displayed the characteristic loss of at least one methoxyl group at M-32 *m*/*z*, and NMR analysis confirmed the presence of two equivalent methoxyl groups (Fig. 6D). Based on these data and following from the assignment of peak 6, this carotenoid is proposed to be 3,3′-dimethoxy-2,3,2′,3′-tetradehydro-β,β-carotene-4,4′-dione (Fig. 5).

Model of plumage reflectance

The reflectance spectra of normal, burgundy, male *X. punicea* feathers showed a strong, long wavelength reflectance beginning at 600 nm and peaking above 700 nm, and a relatively minor short wavelength reflectance peak at ~390 nm (Fig. 3B). The aberrant orange *X. punicea* feathers showed a reflectance of a similar shape but with the long wavelength reflectance beginning at ~500 nm and peaking by 625 nm (Fig. 3B).

The relative fractions of the total extracted carotenoid for each peak from the burgundy specimen are (from most to least abundant): peak 4 (28.4%), peak 7 (18.4%), peak 6 (13%), peak 3 (11.1%), peak 2 (8.2%), peak 8 (7.4%), peak 1 (7.4%), and peak 5 (6.1%) (Table 1). The reflectance spectrum predicted based on the combined absorption of the extracted carotenoids in solution exhibited increasing reflectance at wavelengths above 472 nm, peak reflectance by 600 nm, and a strong UV reflectance peak at 350 nm (Fig. 3B). The reflectance spectrum of normal male *X. punicea* showed a strong discrepancy from the predicted reflectance. However, the long wavelength slope of the reflectance spectrum of the aberrant orange *X. punicea* is a much closer to the predicted reflectance spectrum, and shows a ~36 nm bathochromic shift relative to the extracted carotenoids in solution (Fig. 3B). The extracted carotenoid reflectance prediction shows a strong UV reflectance that is absent in both normal and aberrant *X. punicea*.

Based on the midpoint of the slopes of the long wavelength reflectance peaks of the normal burgundy and aberrant orange feathers (~59% normalized reflectance), the *X. punicea* carotenoids showed a ~93 nm bathochromic shift in reflectance due to conjugation *in situ* from the unconjugated state in solution or the aberrant orange feathers (Fig. 3B). Interestingly, the ultraviolet reflectance peak showed a more modest bathochromic shift of ~40 nm vs. the modeled reflectance of the isolated pigment absorbances, and no noticeable shift vs. the orange feather reflectance spectrum. The bathochromic shift of normal burgundy male *X. punicea* cannot be a result merely of pigment concentration because pressure or heat treatment reduced this shift without changing pigment concentration. Furthermore, Andersson et al. [24] document that a red bathochromic shift in reflectance due to high concentration of normally yellow carotenoids in the Red-shouldered Widowbird (*Euplectes axillaris*) results in a decrease in the slope of the long wavelength reflectance function. This difference in slope is conspicuously absent from the reflectance of normal burgundy male or aberrant orange *X. punicea*, which have the same slope as the reflectance prediction for the solution of extracted carotenoids (Fig. 3B).

Discussion

We have identified eight main carotenoid pigments in the plumage of male *X. punicea*, including six novel, ring-substituted, methoxy-carotenoids (Fig. 5) that to our knowledge have not been previously identified in avian plumage or any other organism. It should be emphasized that the positions of the functional groups in the structures of the proposed novel carotenoids cannot be assigned unambiguously without further analysis. However, the present assignments represent the most likely candidates based on known metabolic conversions of hydroxy-xanthophylls in other avian species [15,25–27].

Methoxy-carotenoids are previously known from cyanobacteria [28], and sponges [29–31]. More recently, 3-methoxy-zeaxanthin has been reported in the human macula [23]. Although the metabolic origins of 3-methoxy-zeaxanthin in the human macula are unknown, Bhosale et al. [23] suggest that it may by formed by either O-methylation of dietary zeaxanthin, or reductive O-methylation of 3′-oxylutein. Like other animals, sponges cannot synthesize carotenoids, but they do make metabolic changes to carotenoids acquired from their diets. Interestingly, poriferan methoxy-carotenoids, such as aaptopurpurin (3-methoxy-β,χ-carotene), are also associated with the production of violet or purple colors. The metabolic production of these novel carotenoids from dietary precursors would require an enzymatic reaction that is not common in eumetazoan or bilaterian animals. However, methoxy-carotenoids found in sponges (Porifera) and the human macula document that enzymatic methoxylation of the 3-position of a carotenoid β-ring from a dietary precursor is physiologically possible in animals [23,29–31].

The predicted reflectance spectrum of male *X. punicea* based on the extracted carotenoid pigments failed to accurately capture two key features of the observed reflectance spectrum of the plumage: the position of the slope of increasing long wavelength reflectance (above 600 nm) and the amount of UV reflectance. Furthermore, the aberrant orange specimen displayed a very similar pigment profile to those of the two characteristic burgundy specimens indicating that these unique carotenoids are not solely responsible

for the color of *X. punicea*. Völker [5] described the rapid change in color of male *Xipholena* feathers from burgundy to orange produced by pressure or heat treatment. He reported that aberrant orange museum specimens were produced accidentally by exposure to excess heat while drying specimens in the field, and that these plumage variants not natural.

There are at least two possible explanations for the color variation in *X. punicea*. First, there could be additional pigments, such as eumelanins or phaeomelanins, in the feathers that were not extracted and identified. However, early workers have reported that melanins are absent in the *Xipholena* plumages [4,5,32–34]. Following treatment with acidified pyridine in our experiments, the *Xipholena* feathers appeared completely white in color, indicating that there is little or no melanin present [11]. Furthermore, when the feather extract was partitioned with MTBE and water, the aqueous phase was colorless, indicating that the feathers did not likely contain phaeomelanin, which would potentially be extracted via the same procedure used in this work [2]. Therefore it is unlikely, that melanin pigments are present. More likely, as suggested previously [24,35,36], the extracted carotenoids could have substantially different absorbance properties *in situ* within feather β-keratin than in solution. The comparisons of the reflectance spectra of burgundy and aberrant orange feathers to the predicted reflectance based on the extracted pigment absorbances and abundances supports the conclusion that the carotenoids producing the normal feather color are conjugated to proteins or other molecules within the feathers, and that the orange color of the feathers from the aberrant specimen are the result of a partial disruption of this conjugation. This situation is not well described for many other plumages with carotenoid pigmentation. For example, the reflectance spectrum of the lutein pigmented throat patch of female *Icteria virens* conforms closely (within 5 nm) to the expectation based on lutein absorbance in ethanol solution [37]. However, lutein does not contain the distinctive methoxyl groups found in these *X. punicea* pigments. It is likely that these moieties facilitate interactions with β-keratin or other unidentified feather proteins in unique ways analogous to the properties of astaxanthin in crustacyanin proteins in Crustacea [38,39]. It is important to note, however, that these findings do not support the hypothesis by Brush [1] that the observed reflectance is a combination of common carotenoid and a structural blue color produced by light scattering from air cavities in the β-keratin of barb medullary cells.

Early German researchers documented that, unlike the diffuse carotenoid pigments within feathers in other birds, the carotenoids in the feathers of *Xipholena* and *Cotinga* occur in microscopic granules [4,5,32–34]. These authors also documented the unusual pressure sensitivity of these colors. Görnitz and Rensch [4] and Völker [5] all rejected any role of spongy medullary structural component in *Xipholena* that was later proposed by Brush [1]. Völker [5] also concluded that the carotenoids were not bound to proteins as in lobsters, but that an unknown, pressure sensitive microstructure must occur above the yellow–red carotenoid pigments to create the dark red or violet plumage color. In other later work Völker [36] proposed that rhodoxanthin in fruit doves (*Ptilinopus*) could produce feather colors ranging from purple to blue due to interactions with keratins. He further reported that these different colors could be reproduced by subjecting rhodoxanthin in solution to various solid phase media which he hypothesized the feather proteins replicated. Mattern and Völker [32] went further in hypothesizing that *Xipholena* and *Cotinga* carotenoid granules occur in a keratin matrix with fat globules and a watery layer. The carotenoid granules in the "hard" keratin matrix consist of a water-containing gel of "soft" keratins filled with fatty material. The protein gel has a lamellar or fibrillar structure. The spatial orientation of the pigment molecules between layers influences their absorption. Many of these works focused on why these carotenoid plumage colors were so dark, especially in the shiny blackish body plumage of the male White-tailed Cotinga *Xipholena lamellipennis*. Schmidt [34] focused on the light scattering and polarizing optical effects of the carotenoid pigment granules in *X. lamellipennis*. While the specific details of the color production hypotheses proposed in these early works remain difficult to interpret, these papers document that the burgundy and purple carotenoids of *Xipholena* and *Cotinga* exhibit a microscopic granular organization that is distinct from other bird feathers, and that these granular structures appear to play a role in the large bathochromic shift of the pigments *in situ*, and the unusual pressure and heat sensitivity of these feather colors. Increasing pressure could result in structural changes to the keratin fibers and other components of the feathers, analogous to phase changes induced by high pressure in crystalline solids. These structural changes could be envisioned to have an impact on the transition dipoles of the bound carotenoids and lead to shifts in their absorption spectra. The identification of unique, cyclic, methoxy-carotenoids in *X. punicea* indicates that the novel molecular structures of these carotenoids is likely to be critically involved in the formation of these apparently unique granular structures. The molecular content, development, and photobiological function of these pigment granules should be the subject of future work on the distinct color of *Xipholena*, *Cotinga*, and perhaps other birds.

Like most other cotingas, *X. punicea* is an obligate frugivore, occasionally consuming insects [40]. The presence of very similar pigment compositions in two burgundy male *X. punicea* of different provenance – a century old wild bird and a recent zoo specimen – confirm that the observed pigments are neither specific to the exotic diet of wild plants or contaminants from a domestic situation. Common carotenoids reported in the avian diet include lutein, β-carotene, β-cryptoxanthin, and zeaxanthin [3]. All of these molecules except β-carotene contain hydroxyl groups at the 3-carbon position, and in the cases of lutein and zeaxanthin, at the 3′-carbon position. The *o*-methyltransferase enzyme responsible for the methylation of hydroxyl groups in the purple bacteria is reported to have a high-degree of sequence similarity with eukaryotic *o*-methyltransferases [41]. Therefore, it is possible that the methoxyl functional groups proposed here derived from enzymatic methylation of these common dietary carotenoids before deposition in the plumage (Fig. 1). Occurrence of a methoxyl group at the 2-carbon position is less likely, as hydroxyl groups in this position are uncommon in nature, and a gene for an enzyme responsible for a 2-carbon hydroxylation reaction has not been reported [41]. Moreover, the β-4-oxygenase enzyme has been reported in birds, specifically those displaying red plumage [11,15,22,26,27]. The *X. punicea* pigments reported here which have a β-carotene-like chromophore (HPLC peaks 1, 2, 3, 5, 6, and 8) are confirmed to contain carbonyls at the carbon-4 and 4′-positions. The remaining HPLC peaks which have an α-carotene chromophore (peaks 4 and 7) have a carbonyl at the 4-carbon position only.

It is possible that the novel carotenoids of *X. punicea* are derived metabolically from common dietary carotenoids by the conversion of hydroxyl groups at the 3- and 3′-positions to methoxyl groups (Fig. 7). A metabolic model could generate predictions about the relative frequency of occurrence of the carotenoids in the feather extracts (Table 1) using relative probabilities for oxygenation, methylation, and dehydrogenation reactions. For the burgundy specimens, the two β,ε-carotene derivatives – peaks 4 and 7 – were the most abundant carotenoids extracted from *X. punicea*, and constituted nearly half (46.8%) of total carotenoid content of the feathers (Table 1). Of these two, 3′-hydroxy-3-methoxy-β,ε-carotene-4-one (peak 4) was more than 50% more common than 3′-hydroxy-3-methoxy-2,3-didehydro-β,ε-carotene-4-one (peak 7). Peak 4 may be derived from dietary lutein via oxygenation of the 4-position of the β-ring to yield α-doradexanthin, followed by methylation

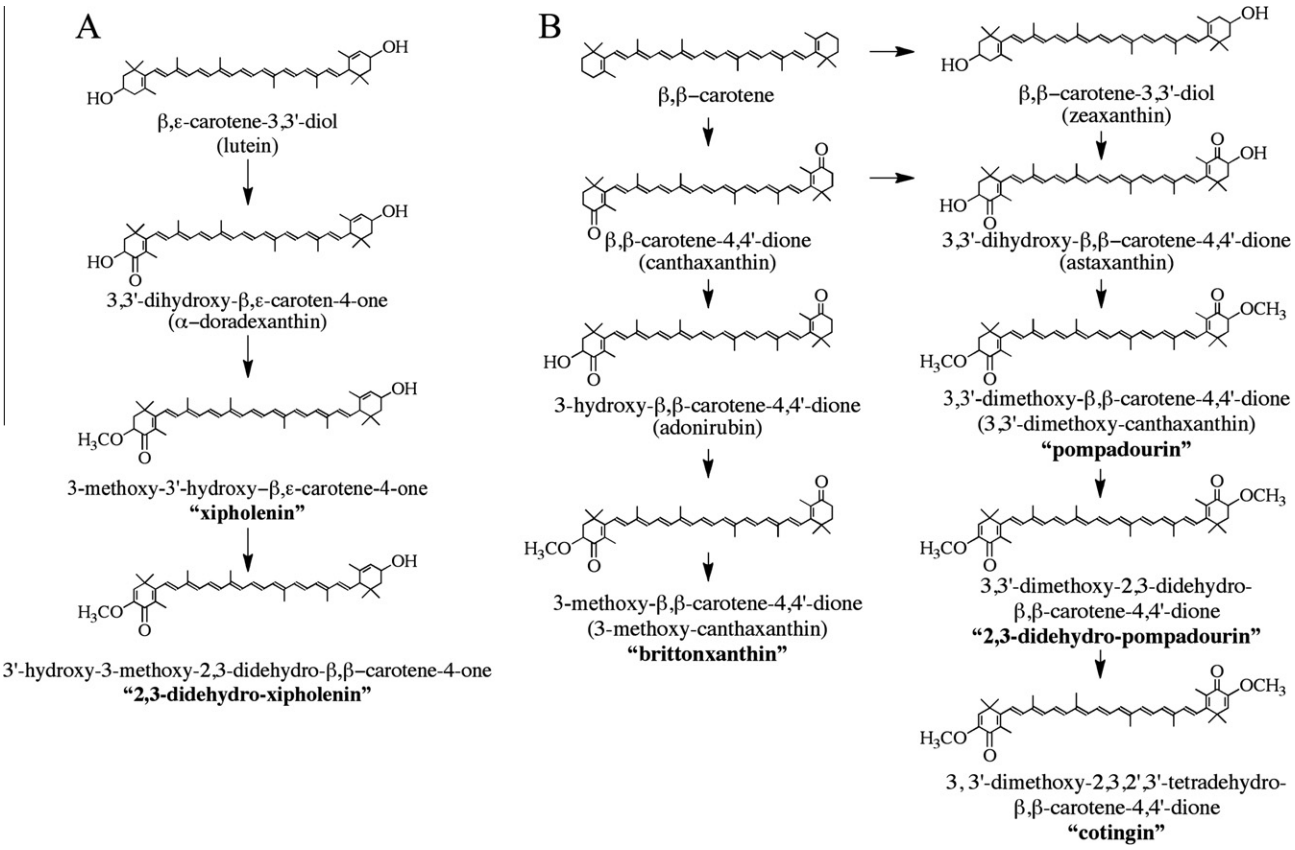

Fig. 7. Proposed metabolic pathways for the derivation of observed carotenoids from dietary precursors. (A) The methoxy-β,ε-carotenes are likely derived from dietary lutein via α-doradexanthin through methylation of the 4-position hydroxyl (peak 4), and dehydrogenation of the 2,3-position of the β-ring (peak 7). (B) The methoxy-β,β-carotenes are likely derived from canthaxanthin via adonirubin and astaxanthin. Peak 2 is likely produced by the methylation of the hydroxyl of adonirubin, and peak 3 by the methylation of both hydroxyls of astaxanthin. Peaks 6 and 8 are then produced by the successive dehydrogenation of the 2,3- and the 2′,3′-positions of the β-rings of peak 3.

of the hydroxyl group in the 3-position of the β-ring (Fig. 7A). Peak 7 could be derived from peak 4 by further dehydrogenation of the β-ring. The 60:40 ratio of these peaks indicates that the hydrogenated form of the β-ring is slightly favored over the dehydrogenated form (Fig. 7A).

The remaining carotenoids are all β,β-carotene derivatives. The data in Table 1 reveals that 3,3′-dimethoxy-canthaxanthin (peak 3) was more abundant than the singly methoxylated 3-methoxy-canthaxanthin (peak 2) or the doubly methoxylated 3,3′-dimethoxy-2,3,2′,3′-tetradehydro-β,β-carotene-4,4′-dione (peak 8). Peak 3 was slightly less abundant than 3,3′-dimethoxy-2,3-didehydro-β,β-carotene-4,4′-dione (peak 6). Canthaxanthin and astaxanthin, which are well known from many other bird plumages, were the least abundant in *X. punicea*, constituting 13.5% of total carotenoid content. The methoxy-carotenoids of *X. punicea* are likely derived from canthaxanthin, adonirubin, and astaxanthin via methylation of the 3- and 3′-position hydroxyls, and subsequent dehydrogenation of the 2,3- or 2′,3′-carbons in the methylated β-rings (Fig. 7B). The relative abundance of the different carotenoids given in Table 1 shows that methylation of 3 and 3′ hydroxyls is highly favored, and that dehydrogenation of the methylated β-rings which converts compounds 3–6 and 6–8 occurs at a rate sufficiently slow to maintain a sizeable amount of the respective precursor molecules.

Although the carotenoid structures proposed here are consistent with all of the chemical and spectroscopic analyses performed to date, it remains unclear why the molecular structures assigned to HPLC peaks 6, 7, and 8 give rise to such long retention times characteristic of highly polar carotenoids. One possibility is that initially upon extraction and during analysis by HPLC, these molecules contain one or more additional polar functional groups that may end up be cleaved during ionization and analysis by mass spectrometry. If this were the case, the peaks in the mass spectra assigned as parent ions for HPLC peaks 6, 7 and 8, may instead represent those of daughter ions. Polar groups attached to carotenoids through ester or glycoside linkages have been reported [42,43], but saponification did not indicate ester linkages were present in any of the molecules. Nevertheless, we have attempted to identify such functional groups through various chemical means and through precursor ion mass spectral analyses, but so far to no avail. Further examination of this issue is ongoing.

In addition to the three species of *Xipholena*, similar vibrant colors are present in the plumages of males of several other cotinga genera including *Cotinga cotinga*, *Haematoderus militaris*, and *Querula purpurata*. A lighter violet pigmentary color is found in males of the three species of Neotropical purpletufts (*Iodopleura*, Tityridae). Elsewhere in birds, similar plumage colors to *Xipholena* are present in southeast Asian orioles (e.g. *Oriolus cruentus*; Oriolidae), and the southeast Asia broadbill *Cymbirhynchus macrorhynchos* (Eurylaimidae). Three species of closely related *Eurylaimus* broadbills (Eurylaimidae) have light violet plumage colors similar to a pale male *Iodopleura*. Various Asian and Australopapuan species of fruit pigeon (e.g. *Ptilinopus magnificus*; Columbidae) exhibit similar burgundy, purple, violet, and pink pigmentary colors that have been identified as rhodoxanthin [44], but this identification should be confirmed with modern techniques. The plumage colors of these other birds may be additional, independent origins of the metabolic production and plumage deposition of methoxy-ketocarotenoids in avian plumage outside of *Xipholena*.

The origin of novel carotenoid pigments in *Xipholena* and potentially other lineages raises an interesting question of why avian

carotenoid pigment metabolism evolves for coloration. Most research in this area assumes that the precursor carotenoids are rare in the diet, and that plumage brilliance indicates dietary and individual quality [3,45]. The honest signaling hypothesis, however, cannot explain why metabolic transformations of these molecules evolve. It has been hypothesized that these metabolic transformations are themselves physiologically costly, but there is no direct evidence to support this. Conversely, there are many reasons to expect that competing males will evolve physiological transformations of dietary carotenoids as a mechanism to undermining signal honesty [46,47].

All the families or genera in which these purple or violet carotenoid plumage colors occur are highly frugivorous: cotingas (Cotingidae), broadbills (Eurylaimidae), Old World orioles and figbirds (Oriolidae), and fruit pigeons (Columbidae). In none of these groups are carotenoid precursors likely to be limited in the diet. The majority of fruits analyzed are characterized by relatively large amounts of β-carotene, β-cryptoxanthin, lutein, and zeaxanthin [48]. It is unlikely that these frugivorous birds could obtain enough calories to survive without acquiring more than enough carotenoids to pigment their feathers. It appears that the novel metabolic carotenoid transformations found in *Xipholena*, and potentially other avian groups, have evolved under exactly the conditions that are not expected to foster the evolution of signal honesty – abundance of easily accessible dietary carotenoids. Alternatively, metabolic transformations of dietary carotenoids in plumage signaling may have evolved because the colors add to the diversity of plumage signaling (Stoddard and Prum, manuscript in Review), and are attractive to females through arbitrary Fisherian sexual selection process or sensory bias [46,47].

Following Görnitz and Rensch [4], the novel methoxy-carotenoids should be recognized with unique chemical names. We propose that the most abundant carotenoid in *X. punicea* – 3′-hydroxy-3-methoxy-β,ε-carotene-4-one (peak 4) – be called "xipholenin" based on the genus name *Xipholena*, which is derived from Greek meaning "sword upper arms" referring to the specialized upper wing coverts of *X. punicea* [49] (see Fig. 1). The closely related peak 7 can be called "2,3-didehydro-xipholenin." We propose that the most polar carotenoid in *X. punicea* – 3,3′-dimethoxy-2,3,2′,3′-tetradehydro-β,β-carotene-4,4′-dione (peak 8) – be called "cotingin" after Görnitz and Rensch [4]. We further propose that peak 3 – 3,3′-dimethoxy-canthaxanthin – be called "pompadourin" based on the common name of *X. punicea* – the Pompadour Cotinga, which is a tribute to Jeanne Antoinette Poisson Marquise de Pompadour (1721–1764) who was the mistress of King Louis XV of France whose favorite color was purple [49]. Peak 6 can then be referred to as "2,3-didehydro-pompadourin." Lastly, we propose that peak 2 – 3-methoxy-canthaxanthin – be named "brittonxanthin" in honor of Professor George Britton whose contributions to the field of carotenoids in general, and this work in particular, are immeasurable.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2010.08.006.

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