

Supplemental Information:

Molecular Diversity, Metabolic Transformation, and Evolution of Carotenoid Feather Pigments in Cotingas (Aves: Cotingidae)

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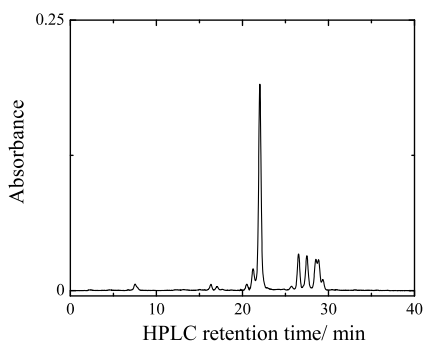
Pigment identifications

Here we provide detailed descriptions of the original data supporting the identifications of the carotenoid pigments extracted from the feathers of cotingas.

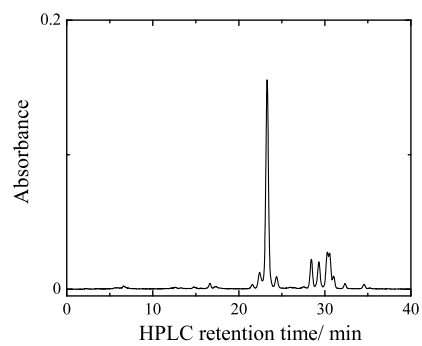
Carpornis cucullatus, *Ampelion rufaxilla*, *Ampelioides tschudii*, *Phibalura flavirostris*, *Snowornis subalaris*, and *Tijuca atra*,

While there is some variation in the retention times of the HPLC peaks associated with the major pigments in these six individuals, it is clear from the chromatograms and absorption spectra of the peaks that these birds share a common pigment profile. Based on the HPLC retention time and absorption spectrum of the pigment eluting at ~22 min in the *Tijuca atra* feather extract chromatogram (Fig. S1), the primary pigment in these feathers is lutein, and the less abundant peaks appearing primarily at longer retention times are cis isomers of lutein created during the extraction procedure. Thus, lutein is identified as the main pigment in the plumage of all six specimens. The peak occurs at 24 min in *S. subalaris* (Fig. S2), 20 min in *P. flavirostris* (Fig. S3), 18 min in *C. cucullatus* (Fig. S4), 20 min in *A. rufaxilla* (Fig. S5), and 21 min in *A. tschudii* (Fig. S6).

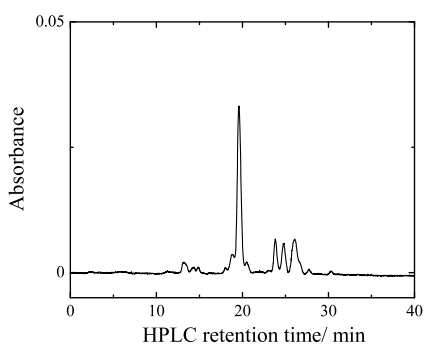
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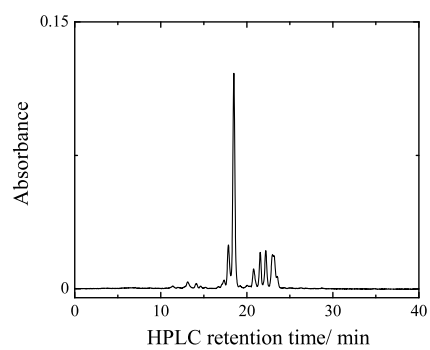
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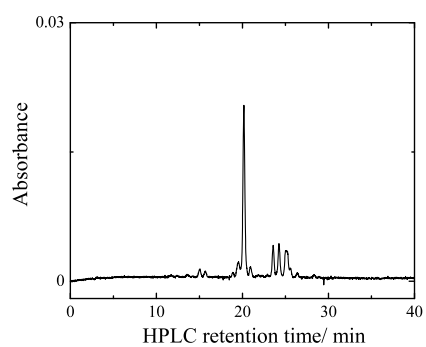
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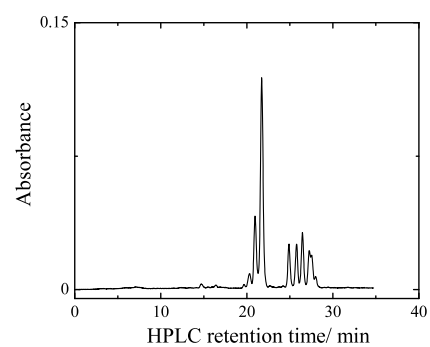
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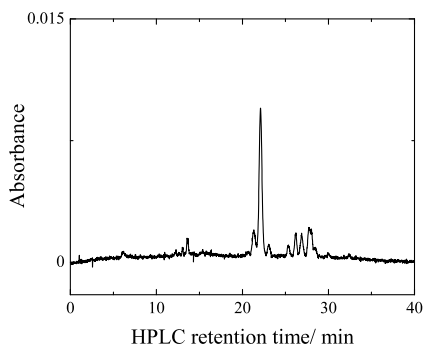
Figures S1-S6. HPLC chromatograms of *Tijuca atra* (S1), *Snowornis subalaris* (S2), *Phibalura flavirostris* (S3), *Carpornis cucullatus* (S4), *Ampelion rufaxilla* (S5), and *Ampelioides tschudii* (S6).

Pipreola aureopectus, Pipreola formosa, and Pipreola chlorolepidota

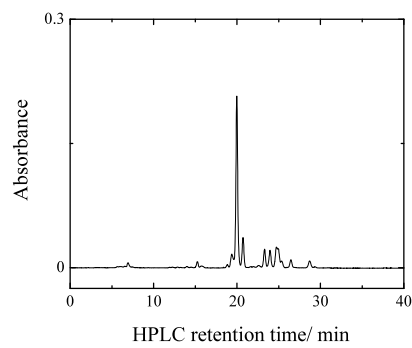
Feathers from two different specimens of *P. aureopectus* were analyzed separately. The yellow contour feathers of *P. aureopectus* (YPM 40970) (Fig. S7) showed the characteristic chromatogram of lutein (21 min) and lutein isomers (25-29 min). The yellow belly feathers of a second specimen of *P. aureopectus* (AMNH 155775) showed the same characteristic chromatogram (Fig. S8) of lutein (26 min) and lutein isomers (31-33 min), along with a significant zeaxanthin peak which eluted just after lutein at ~27 min. The HPLC chromatograms of the yellow belly feathers (Fig. S9) and orange throat feathers (Fig. S10) of *P. formosa*, as well as the orange throat feathers of *P. chlorolepidota* (Fig. S11), have similar pigment profiles with two peaks eluting at ~24 and 25 min. These are also identified as lutein and zeaxanthin, respectively.

Additionally, the HPLC of *P. chlorolepidota* and *P. formosa* included a third pigment which eluted at ~20 min. The absorption spectrum of this peak has a spectrum consistent with that of an α -carotene chromophore. The pigment had similar retention time to α -doradexanthin but too much vibronic structure to be this molecule. Therefore, further analysis is precluded due to lack of material, and therefore the peak remains unidentified.

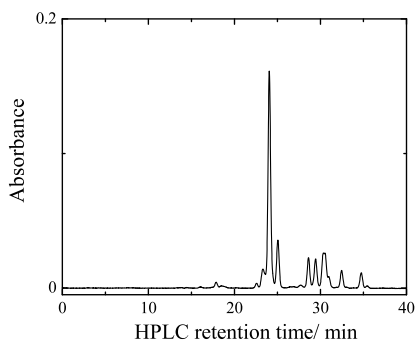
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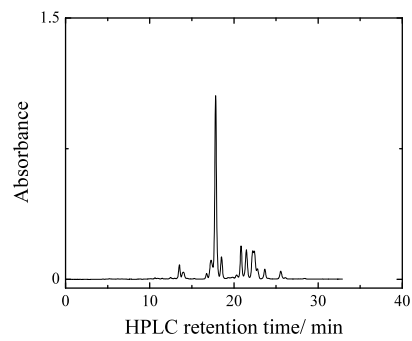
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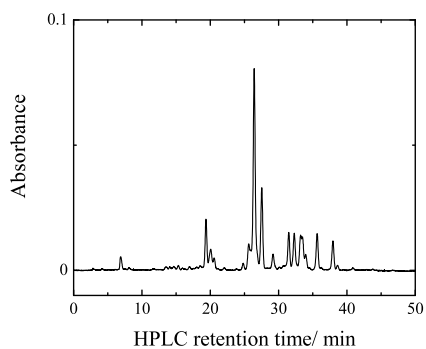
S9



S10



S11



Figures S7-S11. HPLC chromatograms of yellow *Pipreola aureopectus* (YPM 40970)(S7), *Pipreola aureopectus* (AMNH 155775) (S8), yellow belly (S9) and orange throat feathers of *Pipreola formosa* (S10), and orange throat feathers of *Pipreola chlorolepidota* (S11).

Pipreola whitelyi

Based on the retention time (Fig. S12) and absorption spectrum of the peak eluting at 25 min, as well as the occurrence of lutein in other *Pipreola* species, this peak is identified as lutein. The peak eluting at 20 min has a retention time and absorption spectrum similar to that of α -doradexanthin. At this time further analysis is precluded by insufficient material.

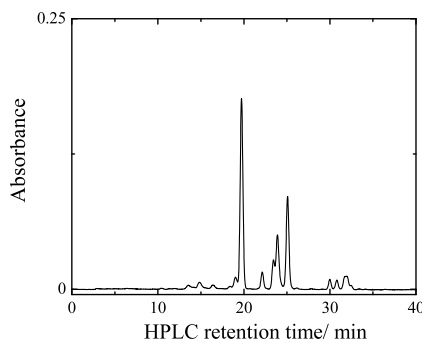


Figure S12. HPLC chromatogram of *Pipreola whitelyi*.

Procnias tricarunculata

The normal-phase HPLC chromatogram, as well as the absorption spectra of the two main peaks, observed at ~8 and 12 min (Fig. S13), are consistent with those observed in the red back feathers of *Tangara chilensis*, which were identified as canary xanthophyll A and B, respectively, by mass spectrometry (data not shown). Therefore, the two main pigments of *P. tricarunculata* are also identified as canary xanthophyll A and B.

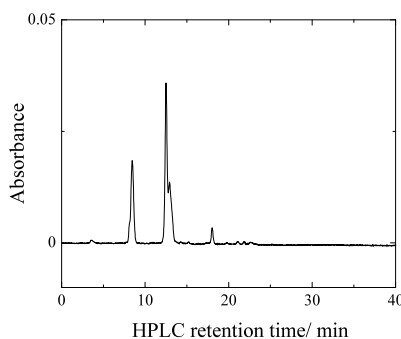


Figure S13. HPLC of *Procnias tricarunculata* feather extract.

Pyroderus scutatus scutatus and *Pyroderus scutatus occidentalis*

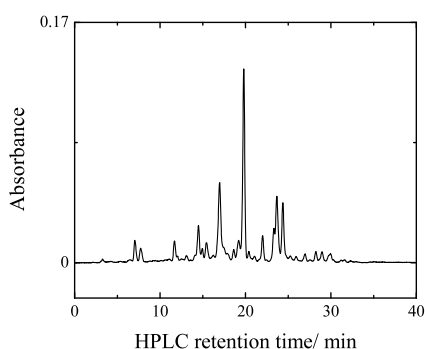
In both subspecies of *P. scutatus* there was a significant amount of coloration remaining in the feathers even after 3 hr of heat treatment in acidified pyridine. Some of this coloration is likely due to the presence of melanin, although there remained a reddish hue, which may be caused by a residual carotenoid or other pigmentation. In both subspecies, canthaxanthin and a canthaxanthin cis-isomer were observed eluting at approximately 6 min.

The peak at ~17 min in *P. s. scutatus* (Fig. S14) and 14 min in *P. s. occidentalis* (Fig. S15) displays an absorption spectrum with a high degree of vibronic resolution, consistent with

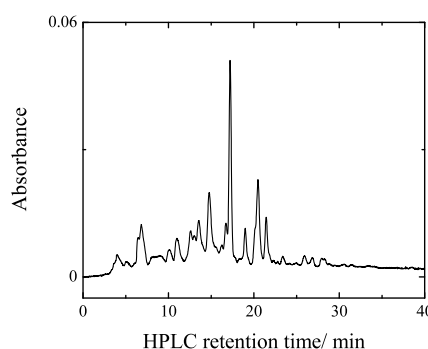
that of canary xanthophyll A. The mass spectral analysis of this pigment collected from *P. s. scutatus* yielded a value of 566 m/z, which confirms the identification of this peak as canary xanthophyll A. The peak observed at 20 min in *P. s. scutatus* and 17 min in *P. s. occidentalis* has a broad absorption spectrum consistent with the presence of one conjugated carbonyl, and shifts to a lutein-like (α -carotene) chromophore upon chemical reduction with sodium borohydride. Mass spectral analysis reveals a mass of 582 m/z. Based on these observations, this peak is identified as α -doradexanthin.

The peak at 23 min in *P. s. scutatus* shows the same mass spectral data and absorption spectrum, along with a characteristic cis peak at ~ 355 nm, and is therefore identified as *cis*- α -doradexanthin. The peak at 24 min in *P. s. scutatus* and 20 min in *P. s. occidentalis* is identified as lutein based on co-chromatography with a known standard.

S14



S15



Figures S14-S15. HPLC chromatograms of *Pyroderus scutatus scutatus* (S14) and *Pyroderus scutatus occidentalis* (S15).

Phoenicircus carnifex

Mass spectral analysis performed on the three prominent peaks appearing at 14, 15 and 17 min (Fig. S16) revealed the same mass of 562 m/z. Furthermore, the absorption spectra of all three show a λ_{max} in ethanol of 495 nm. Thus, these three pigments are identified as rhodoxanthin and rhodoxanthin cis isomers. This finding is consistent with previous work by Volker, who proposed that rhodoxanthin was the primary pigment in the red feathers of this species and the closely related *Phoenicircus nigricollis* (Hudon et al., 2007).

The pigment eluting at 27 min is identified as lutein based on mass spectral analysis, HPLC retention time and absorption spectrum compared to a known standard. Saponification of the total extract was conducted using the method outlined by Eugster (Eugster, 1995). Esterified lutein (obtained from Advanced Medical Nutrition, Inc.) was saponified alongside the feather extract to ensure the efficacy of the reagents. No evidence of ester linkages in the pigments from *Phoenicircus carnifex* were found.

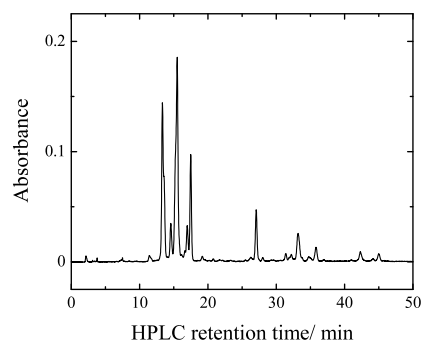


Figure S16. HPLC of *Phoenicircus carnifex* feather extract. The chromatogram was recorded using a detection wavelength of 440 nm.

Rupicola rupicola

The absorption, molecular mass, and retention time of the peak eluting at 7 min is consistent with canthaxanthin. Based on this identification and the absorption spectrum, the next immediate peak is identified as the central *cis*-isomer of canthaxanthin.

The peak which eluted at 15 min was resolved into two separate peaks by reversed phase chromatography. The lesser of these two pigments has an absorption spectrum and molecular mass consistent with canary xanthophyll A. Sodium borohydride reduction of the more abundant pigment yielded an α -carotene-like chromophore, and a spectral shift consistent with one carbonyl in conjugation. Based on the HPLC retention time, mass spectral data, and absorption spectrum, it is very likely that this peak is that of xipholenin (LaFountain et al., 2010). The presence of a methoxyl group was confirmed by $^1\text{H-NMR}$.

The peak eluting at 17 min has an absorption spectrum and molecular mass consistent with those of α -doradexanthin. Sodium borohydride reduction produced an α -carotene-like chromophore and a red-shift consistent with only one conjugated carbonyl. Mass spectral analysis of the reduction product shows a gain of 2 mass units upon chemical reduction, which is consistent with the presence of only one carbonyl and provides further support for this identification. Further supporting evidence for an identification as α -doradexanthin was obtained by $^1\text{H-NMR}$, which showed evidence of both β - and ϵ -rings.

The co-eluting peaks at 22 min were also resolved into two peaks using reversed phase HPLC. The molecular masses and absorption spectra of these peaks are consistent with *cis*-isomers of α -doradexanthin and lutein.

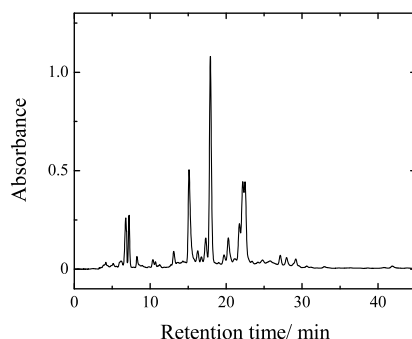
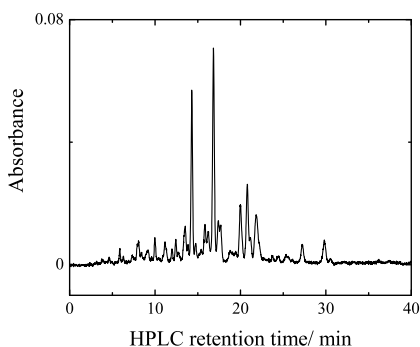


Figure S17. HPLC of *Rupicola rupicola* feather extract.

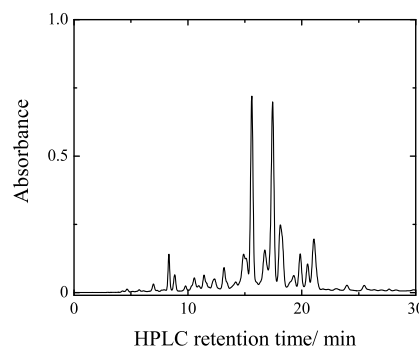
Rupicola peruviana saturata* and *Rupicola peruviana sanguinolenta

The HPLC chromatogram of the extract of orange belly feathers from a *Rupicola p. saturata* (Fig. S18) and the red-orange body feathers of a *Rupicola p. sanguinolenta* specimen (Fig. S19) both showed three major peaks. Two of the largest peaks, which eluted at 14 min in *Rupicola p. saturata* and 16 min in *Rupicola p. sanguinolenta*, were identified as rupicolin as described in detail in the manuscript. Two additional peaks, which eluted 16 min in *Rupicola p. saturata* and at 20 min in *Rupicola p. sanguinolenta*, have the same broad absorption spectrum characteristic of a ketocarotenoid. In both cases, the molecular masses of the pigments were found to be 582 m/z. For further insight into the identities of these peaks, the pigment collected from *Rupicola p. saturata* was chemically reduced in methanol using sodium borohydride, which resulted in a shift of ~12 nm to shorter wavelength, consistent with the presence of one conjugated carbonyl. The reduction product had an absorption spectrum consistent with a β -carotene like chromophore. Based on the absorption spectrum, HPLC retention time relative to known standards, and the identification of rupicolin as another major pigment, this pigment is identified as adonixanthin. The peak eluting at 21 min in *Rupicola p. saturata* is identified as the xanthophyll, zeaxanthin, based on its HPLC retention time and absorption spectrum in ethanol.

S18



S19



Figures S18-S19. HPLC chromatograms of *Rupicola peruviana saturata* (S18) and *Rupicola peruviana sanguinolenta* (S19).

Xipholena punicea*, *Xipholena lamellipennis*, and *Xipholena atropurpurea

The identification of the eight carotenoids of *X. punicea* plumage (Fig. S20) is described in detail in LaFountain et al. (2010). The additional species of the genus *Xipholena*, *X. lamellipennis*, *X. atropurpurea*, *H. militaris*, and *Q. purpurata* display variations of the pigment profile reported for *X. punicea*.

The HPLC chromatograms of the extracted pigments of *X. lamellipennis* (Fig. S21) and of *X. atropurpurea* (S22) showed six major pigments, all of which have broad absorption spectra characteristic of carotenoids with at least one carbonyl in conjugation. The HPLC retention times, relative positions in the chromatogram, and absorption spectra identify these pigments as canthaxanthin (~6 min), 3-methoxy- β,β -carotene-4,4'-dione (brittonxanthin, ~9 min), 3,3'-dimethoxy- β,β -carotene-4,4'-dione (pompadourin, ~11 min), and 3'-hydroxy-3-methoxy- β,ϵ -carotene-4-one (xipholenin, ~17 min). It is possible that the feathers of *X. lamellipennis* also

contain melanin, as they remained quite dark even after ~2 hr of treatment with acidified pyridine.

Haematoderus militaris*, and *Querula purpurata

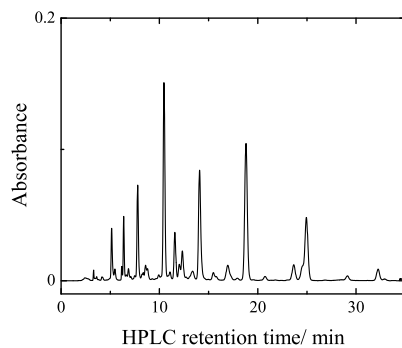
Similarly, the major pigments in the extract from the feathers of *H. militaris* were identified by mass spectrometry and by comparison of the absorption spectra and HPLC retention times with the results from *X. punicea*. The retention times of these pigments vary slightly from those of the *X. atropurpurea* and *X. lamellipennis*, but are consistent with those of *X. punicea*. The peak eluting at ~8 min (Fig. S23) has a molecular mass of 594 m/z, and an absorption spectrum consistent with that of 3-methoxy- β,β -carotene-4,4'-dione (brittonxanthin). The peak eluting at 11 min has a molecular mass of 624 m/z and an absorption spectrum consistent with that of 3,3'-dimethoxy- β,β -carotene-4,4'-dione 3 (pompadorin). The peak eluting at 17 min has a molecular mass of 596 m/z and an absorption spectrum consistent with 3-methoxy-3'-hydroxy- β,ϵ -carotene-4-one (xipholenin). The peak eluting at 21 min has a molecular mass of 622 m/z and an absorption spectrum consistent with 3,3'-dimethoxy-2,3-didehydro- β,β -carotene-4,4'-dione (2,3-didehydro-pompadorin). Rhodoxanthin was also identified at 14 min based on comparison of the retention time and absorption spectrum with a bona fide standard.

Unlike the other Cotingidae specimens, the feathers from *Q. purpurata* retained an orange sheen following 2 hr of heating to 90°C in acidified pyridine. Therefore, after separating the extract from the feathers, fresh acidified pyridine was added and the sample was incubated at 90°C for an additional 2 hr. After this second extraction, the feathers were still orange, and only a small amount of additional pigmentation had been extracted. This subsequent extract was analyzed by HPLC and the pigment profile was found to be identical to the initial extraction.

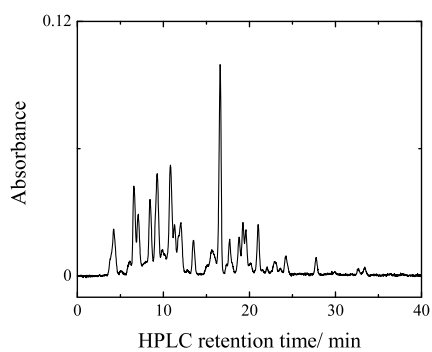
The four major pigments were collected by the HPLC protocol and re-purified by RP-HPLC (Fig. S24). The absorption spectra of these individual peaks were measured in ethanol and found to be consistent with peaks having corresponding retention times in the *X. punicea* extract. The mass spectral data confirmed that the four main pigments of *Q. purpurata* are consistent with four of the major *X. punicea* pigments, including 3-methoxy- β,β -carotene-4,4'-dione (brittonxanthin) at ~7 min, eluting at ~7 min; 3,3'-dimethoxy- β,β -carotene-4,4'-dione (pompadorin) eluting at ~9 min; 3'-hydroxy-3-methoxy- β,ϵ -carotene-4-one (xipholenin), eluting at ~13 min; and 3'-hydroxy-3-methoxy-2,3-didehydro- β,ϵ -carotene-4-one (2,3-didehydro-xipholenin) eluting at ~24 min.

In order to determine whether any of these pigments have ester linkages, saponification was carried out according to the methods of Eugster (Eugster, 1995). The HPLC of the resulting extract showed no indication of esters.

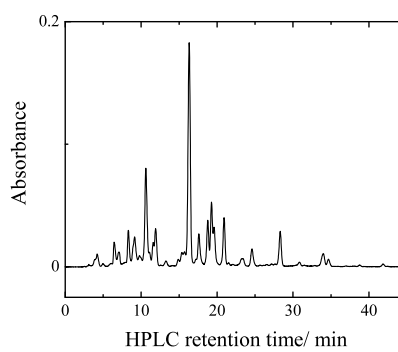
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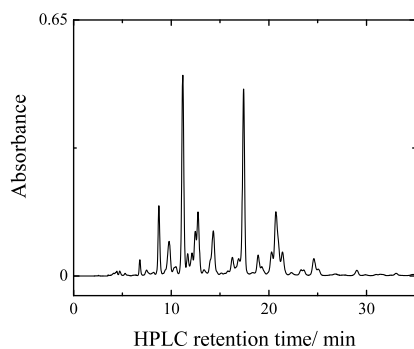
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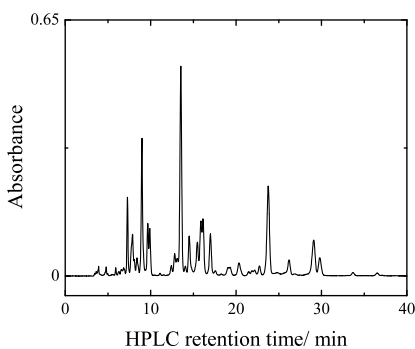
S22



S23



S24



Figures S20-S24. HPLC chromatograms of *Xipholena punicea* (S20), *Xipholena lamellipennis* (YPM 304) (S21), *Xipholena atropurpurea* (S22), *Haematoderus militaris* (S23), and *Querula purpurata* (S24). The detection wavelengths for the chromatograms were as follows: *X. punicea*, 470 nm; *X. lamellipennis*, *X. atropurpurea*, and *Q. purpurata*, 450 nm; *H. militaris*, 440 nm.

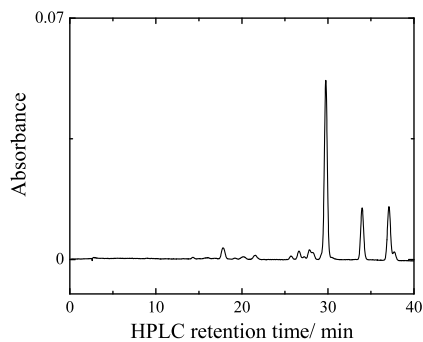
Cotinga cotinga*, *Cotinga maculata*, *Cotinga amabilis*, *Porphyrolaema porphyrolaema*, and *Lipaugus streptophorus

The HPLC chromatograms of the purple breast feathers of *C. cotinga* (Fig. S25), the purple breast feathers of *C. maculata* (Fig. S26), the purple breast feathers of *C. amabilis* (Fig. S27), the purple throat feathers of *P. porphyrolaema* (Fig. S28), and the pink undertail feathers of *L. streptophorus* (Fig. S29) revealed nearly identical pigment profiles, with a single dominant pigment eluting at ~30 min, followed by two minor peaks eluting 2-4 min apart. The absorption spectrum of the third peak clearly showed the characteristic profile of a cis isomer (i.e. an absorption band at ~100 nm blue-shifted from the λ_{\max} (Britton 1995) . In order to determine that the two minor peaks were isomers of the major peak, the major peak from the NP-HPLC of *C. cotinga* was collected, re-purified using a RP-HPLC protocol, heated in acidified pyridine for 30 min, and re-injected into the HPLC run using a NP protocol. The two minor peaks were present in this chromatogram, indicating that they are indeed isomers of the major peak.

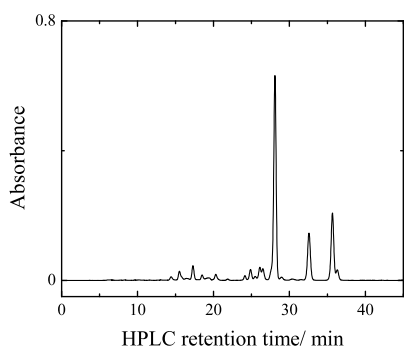
High-resolution mass spectrometry of the major peak observed at ~30 min in the *C. cotinga* HPLC chromatogram (Fig. S25) revealed a molecular mass of 643.3788 with a mass accuracy of 4.7 ppm. This is consistent with a carotenoid mass of 620.387 combined with a sodium adduct as is common in mass spectral analyses. High-resolution mass spectrometry obtained previously on the corresponding peak from *X. punicea* identified it as cotingin with a mass of 620.3865 m/z consistent with an empirical formula of $C_{42}H_{52}O_4$ (0.2 ppm mass accuracy) (LaFountain et al., 2010) . This result supports this assignment of this peak to cotingin.

The extract from the feathers of *L. streptophorus* revealed an additional pigment which had the retention time and absorption spectrum consistent with 2, 3-didehydro-pompadourin (Figure S29, 17 min). It should be noted that *L. streptophorus* has a unique pink color as opposed to the brilliant purple of the other species mentioned above, and therefore is the first instance thus far of cotingin as the primary pigment giving rise to a color other than purple.

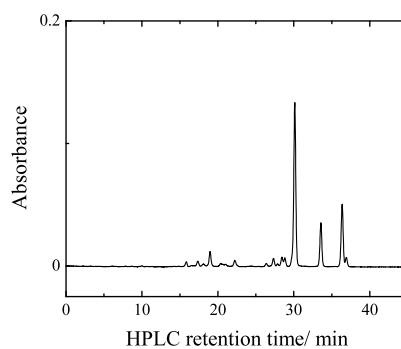
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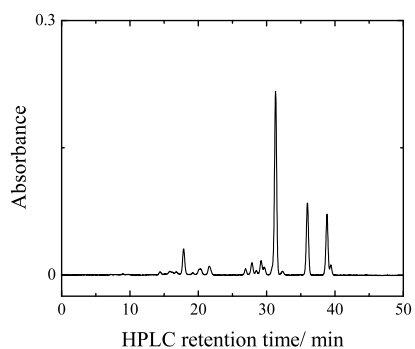
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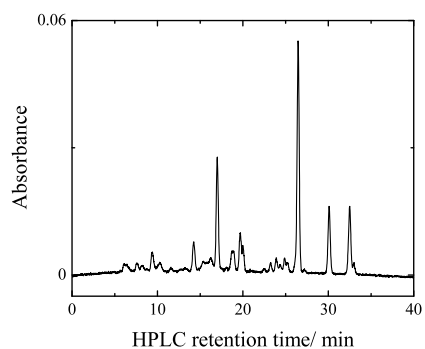
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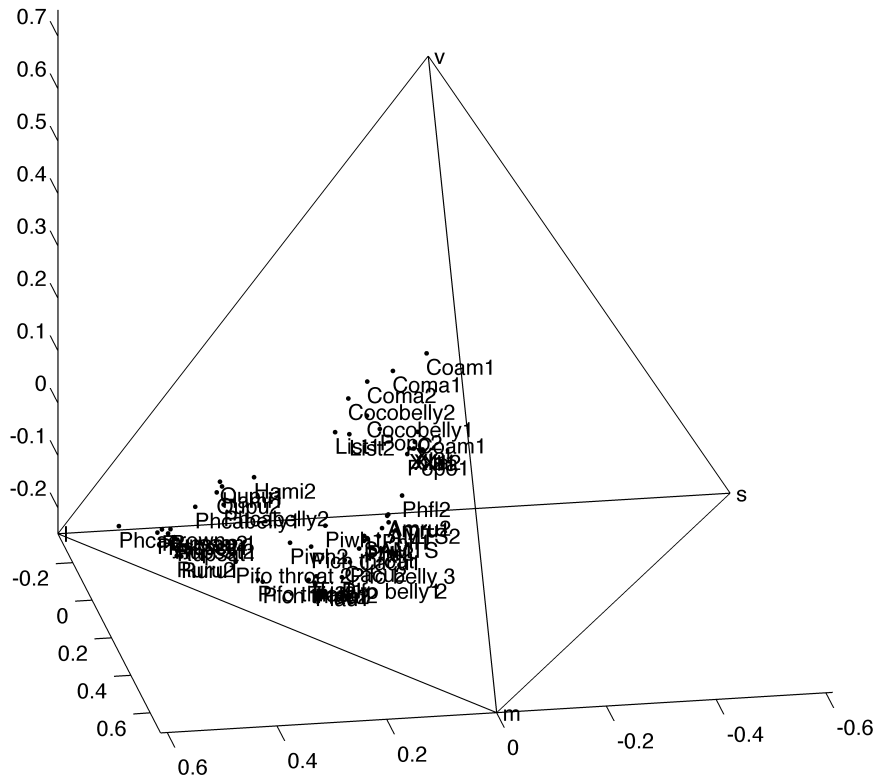
S28



S29



Figures S25-S29. HPLC chromatograms of *Cotinga cotinga* (S25), *C. maculata* (S26), *C. amabilis* (S27), *P. porphyrolaema* (S28), and *L. streptophorus* (S29). *C. maculata* and *C. amabilis* were recorded using a detection wavelength of 450 nm, while all others were detected at 470 nm.



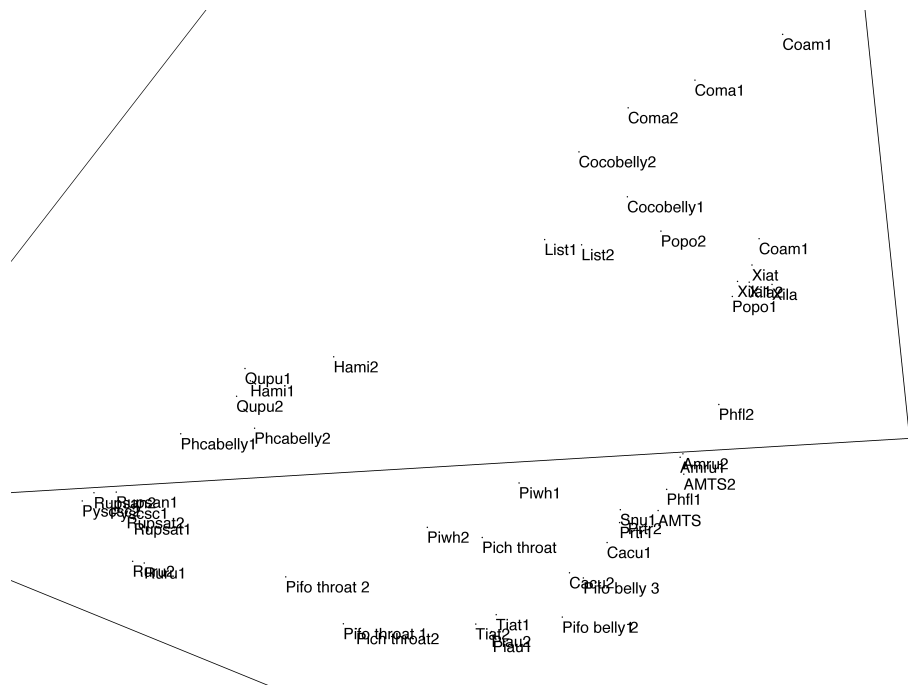


Figure S30. Distribution of the reflectance spectra from 29 cotinga carotenoid plumage patches in the tetrahedral color space. **Top-** Full tetrahedron. **Bottom-** Expanded section of tetrahedron. Each species is plotted with an abbreviation of the first two letters of its genus name followed by the first two letters of its species name. For example *Cotinga amabilis* is Coam. Each species is represented by 1-4 reflectance spectra. Different plumage patches are also numbered.

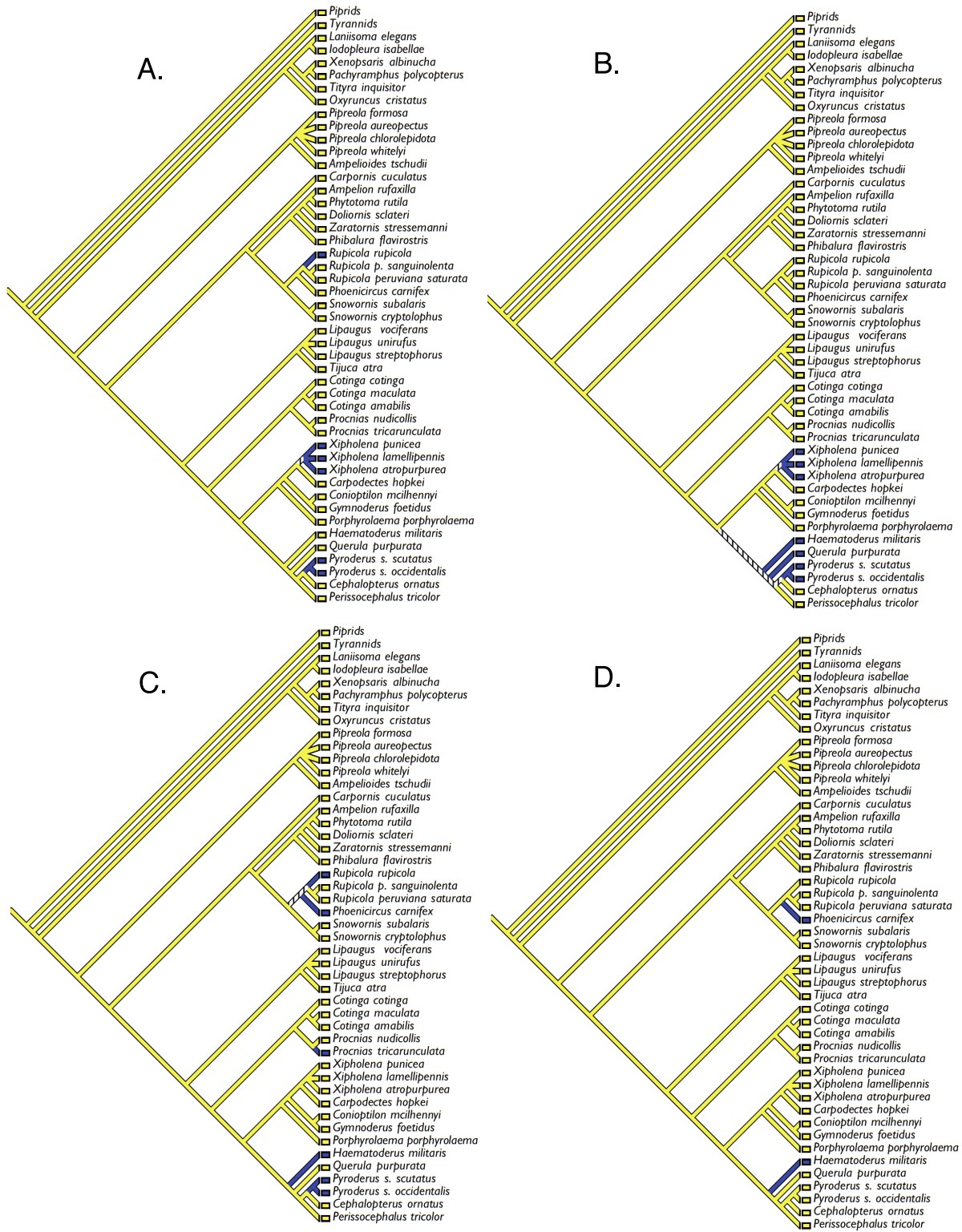


Figure S31. Phylogenetic hypotheses for the evolution of incorporation of (A) β -cryptoxanthin derivatives, and (B) β -carotene derivatives into the plumages of cotingas and

outgroups based on a phylogeny by Tello et al. (2009). Phylogenetic hypotheses for the evolution of metabolic reactions for the transformation of dietary precursor carotenoids in cotingas and their outgroups: (C) hydroxyl dehydrogenation, $K_4 > 0$, (D) C6-hydroxylation, $K_4 > 0$ (or K_4 and $K_5 > 0$) and retro-conversion, $K_6 > 0$.

Table S1

The following tables give the formulae for each relative amount of each molecule in each pathway as a function of the relative equilibrium constants of the forward metabolic reactions.

	Relative amount
Lutein pathway	
lutein	1
α -doradexanthin	K_1
xipholenin	$K_1 * K_2$
2,3-didehydro-xipholenin	$K_1 * K_2 * K_3$
canary xanthophyll A	$2 * K_4$
canary xanthophyll B	$2 * K_4^2$
6-hydroxy- ϵ,ϵ -carotene-3, 3'-dione	$2 * K_4^2 * K_5$
rhodoxanthin	$2 * K_4^2 * K_5 * K_6$
Zeaxanthin pathway	
zeaxanthin	1
adonixanthin	$2 * K_1$
rupicolin	$2 * K_1 * K_2$
astaxanthin	$2 * K_1^2$
3'-hydroxy-3-methoxy-canthaxanthin	$4 * K_1^2 * K_2$
pompadourin	$4 * K_1^2 * K_2^2$
2,3-didehydro-pompadourin	$8 * K_1^2 * K_2^2 * K_3$
cotingin	$8 * K_1^2 * K_2^2 * K_3^2$
β-cryptoxanthin pathway	
β -cryptoxanthin	1
3-hydroxy-echinenone	K_1
adonirubin	K_1^2
brittonxanthin	$K_1^2 * K_2$
β-carotene pathway	
β -carotene	1
echinenone	$2 * K_1$
canthaxanthin	$2 * K_1^2$