Analytical Methods

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Analytical Methods

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PAPER

Non-destructive descriptions of carotenoids in feathers using Raman spectroscopy

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Chemical analyses of pigments in skin, scales, feathers and fur have provided deep insight into the colouration and visual communication strategies of animals. Carotenoid pigments in particular can be important colour signals in birds and other animals. Chromatographic analyses of plumage carotenoids require the destruction of one or more feathers, which has made pigment research on threatened species or museum specimens challenging. Here we show that Raman spectroscopy, coupled with multivariate statistics, can be used to identify the most abundant carotenoid within a single feather barb without sample destruction. Raman spectra from the feathers of 36 avian species were compared to data on pigment presence from high-performance liquid chromatography. Feathers rich with α-doradexanthin, astaxanthin, canary xanthophylls, canthaxanthin, cotingin or lutein were discriminated by subtle shifts in Raman spectral band positions, and by novel bands associated with particular carotenoids. As an example application of this method, we predicted the most abundant carotenoid in the plumage of selected Australian and New Zealand songbirds. α-doradexanthin is predicted in the plumage of *Petroica* robins from Australia, whereas *Petroica* immigrants to New Zealand display a yellow carotenoid that is likely lutein. Raman spectroscopy is useful for non-destructive studies of carotenoids and is well-suited for analysing large ornithological museum collections.

1 Introduction

Many animals use pigments in the integument (i.e. in skin, scales, feathers or fur) for camouflage or visual communication. Classic examples of pigments as visual signals include the black, red and yellow warning stripes across the scales of a coral snake (*Micrurus fulvius*) and the orange and ultraviolet-reflecting scales of sulphur butterflies (*Colias eurytheme*) that advertise individual quality.[1,2] From chemical analyses of integumentary pigments, we have gained deep insight into both how and why animals communicate in colour.[3]

Red, orange, and yellow carotenoid pigments are abundant in organisms, ranging from plants, where they serve accessory photosynthetic roles, to animals, where they can play key roles in sexual advertisement. Studies of carotenoids in animals have provided important insights for fields as diverse as evolutionary biochemistry, nutritional ecology and sexual selection.[4,5] Regarding sexual selection, the red, orange and yellow hues of many bird feathers are pigmented with carotenoids and can be important for mate choice.[6] Several types of carotenoid occur in the dietary items of birds, including yellow lutein in many plants and red astaxanthin in several invertebrates.[7,8] Some birds display dietary carotenoids in their plumages (e.g. European greenfinch *Chloris chloris*, American flamingo *Phoenicopterus ruber*), whereas other species deposit new carotenoids modified from dietary pigments into plumage (e.g. Atlantic canary, *Serinus canaria*).[8-10] Modified carotenoids and their dietary precursors can produce substantially different plumage colours (e.g. lutein vs. cotingin in pompadour cotinga, *Xipholena punicea*).[11] Researchers previously have used mass spectrometry and high-performance liquid chromatography (HPLC) to identify at least 25 carotenoid compounds in feathers from ca. 200 bird species.[5]

Typically, studies assessing bird plumage pigments have relied on destructive sampling of tissue to remove the pigment from the feather matrix for subsequent chemical analysis (i.e. HPLC). This sampling scheme limits our ability to study either threatened species or to make good use of specimens in museum collections, where large-scale tissue collection is discouraged. We sought to test a non-destructive technique, Raman spectroscopy, for identifying the most abundant carotenoid pigment in bird feathers. We aimed to determine if Raman spectroscopy could provide pigment information that may be relevant to evolution, physiology or behavioural studies of birds. Modern Raman spectroscopy is used to study the energy exchanged between laser photons and a target sample, which provides information about covalent bonds and thus about the molecules or minerals in a sample. The mechanism underpinning the brilliant colouration of a carotenoid compound is also responsible for producing a vivid Raman spectrum; all carotenoids have a conjugated backbone, and variations in conjugation length, terminal cyclisation and

functional groups distinguish different carotenoids,[5] because they influence the wavelengths of light absorbed by carotenoids and the strength of vibrations between atoms. In previous studies, both Veronelli et al.[12] and Whitenall et al.[13] related shifts in Raman peak positions to the lengths of conjugated backbones, and more recently, Jehlička et al.[14] used Raman peak positions to distinguish the different carotenoid compositions of microbial cultures. Hence, Raman spectra might be used to identify specific carotenoids.

Raman spectra of carotenoids in feathers were first documented by Veronelli et al.,[12] and more recently Mendes-Pinto et al.[15] showed the influence of binding proteins on both the light-absorption properties and Raman spectra of feather carotenoids in a species of purpletuft (genus *Iodopleura*). Both of these earlier studies affirmed that Raman spectra of feathers vary with carotenoid composition, but no prior investigation has used this technique as a diagnostic tool for identifying carotenoid type. Such an approach requires careful calibration of Raman spectra from feathers having known carotenoid content (i.e. as determined with HPLC). Here we present results from Raman and HPLC analyses on feathers from 36 avian species spanning 18 families, and relate variation in Raman spectra to differences in carotenoid composition. We show that our Raman spectroscopic method accurately predicts the most abundant type of carotenoid in a feather. This *in situ* and rapid method of characterising carotenoids represents a new approach to studying feather pigmentation. We also then provide an application of our method, to a set of colorful feathers for which carotenoid content is not currently known (Method Validation and Method Application sections, respectively). We selected *Petroica* robins and other Australasian songbirds for our example application, because: 1) plumage colouration within Petroicidae presents an interesting evolutionary pattern (detailed in Application), and 2) the types of plumage carotenoids displayed by *Petroica* robins likely correspond to carotenoids in our 36 species calibration set.

2 Method Validation

2.1 Materials

Study feathers had previously been removed from birds during the preparation of osteological specimens at the National Museum of Natural History, Smithsonian Institution (Table 1). Feathers had been stored in darkness for up to 33 years and were chosen to represent a range of carotenoid-consistent colours: red, pink, yellow, orange and purple.

2.2 Data collection

Three Raman spectra were collected from each of the 36 feathers; each spectrum was collected from a different feather barb. Spectra were measured using a Nicolet Almega XR spectrometer (Thermo Electron Corporation, Madison, WI, USA), housed at the Museum Conservation Institute, Smithsonian Institution. Feathers did not undergo any specialised sample treatment and were placed on a microscope stage for analysis. Feathers were probed with a 780 nm 150 mW diode laser, through a 50× Mplan apochromatic objective lens (Olympus, Melville, NY, USA) and 100 μm pinhole aperture (BX51 confocal microscope, Olympus, Melville, NY, USA). Carotenoids have a very broad pre-resonance range,[16,17] and hence the spectra collected with the 780 nm excitation wavelength were analytically useful, and were comparable to spectra collected with 532 nm excitation (532 nm spectra not shown). The green wavelength may be more sensitive to fluorescent impurities (i.e. co-deposited melanin) and thus our study used the less sensitive near infrared wavelength. Future studies may wish to evaluate the benefits of using specific excitation wavelengths for particular feathers. Scattered light was collected with a Peltier-cooled CCD detector and each spectrum was a co-addition of 32 scans across 100–3500 cm^{-1} (2.6–4.9 cm^{-1} spectral resolution). A spectrum of a polystyrene standard was collected at the beginning of each session to track the drift in wavenumber values. The ν_1 mode in polystyrene[18] was 1002.3±1.0 cm^{-1} from all sessions.

After Raman analysis, feather pigments were extracted and analysed with HPLC. Coloured barbs (1–31 mg) were cut from feathers, placed in 8 ml glass vials and immersed in a minimum volume of acidified pyridine.[19] Samples were heated in a 97°C water bath for approximately two hours and then cooled to room temperature. The samples were thoroughly mixed after 2 ml distilled water had been added, and were mixed again after 1 ml of a 1:1 hexane:tert-butyl methyl ether solution had been added. Samples were centrifuged for 5 minutes at 3500 rpm and the colourful supernatant was pipette-transferred to a clean vial and evaporated to dryness under a stream of N_2. The dried pigment was dissolved in 200 μl of acetonitrile:methanol:chloroform (46:46:8, v:v:v), of which 90 μl was transferred to an HPLC insert housed by an amber vial that was sealed with a silicon septum and plastic cap. 50 μl of the solution was injected into a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a YMC C-30 Carotenoid column (5.0 μm particle size; 4.6 mm × 250 mm) and a Waters 2996 photodiode array detector (Waters Corporation, Milford, MA, USA) equipped). Instrument was housed in the School of Life Sciences, Arizona State University. A two-step gradient solvent system with a constant flow rate of 1.2 $ml.min^{-1}$ was used to analyse both polar and nonpolar carotenoids in a single run. The first step was an isocratic elution with 42:42:16 (v:v:v) methanol:acetonitrile:dichloromethane for 11 min; the second step was a linear gradient up to 42:23:35 (v:v:v) methanol:acetonitrile:dichloromethane until minute 20, which was held isocratically until minute 27, at which point we returned to initial conditions and held it through minute 29.5.

2.3 Data analysis

All 108 Raman spectra were combined into a single matrix with the intensity values aligned by wavenumber values. Intensity values outside of the 950–1620 cm^{-1} range were removed and the noise in each spectrum was reduced with first order, 13 point Savitzky-Golay smoothing.[20] Each smoothed spectrum was then baseline corrected using an iterative, second derivative algorithm.[21] The 1420–1485 cm^{-1} region of the smoothed, baseline-corrected spectra was removed (band from β-keratin).[22] The intensity values of each spectrum were then normalised against the minimum and maximum intensity values

Table 1 Species for which we analysed carotenoids using high performance liquid chromatography and Raman spectroscopy.

Species name	Common name	Sex	Catalogue #	Carotenoid[a] (proportions)
Apaloderma narina	Narina trogon	Male	USNM[b] 634596	CA 0.67; AD 0.19; EC 0.1; AS 0.03; αD 0.01
Bombycilla cedrorum	Cedar waxwing	Unknown	USNM 623482	XC 0.7; XB 0.3
Cardinalis cardinalis	Northern cardinal	Male	USNM 643555	αD 0.5; CA 0.2; AD 0.1; AS 0.1; XC 0.1
Cardinalis sinuatus	Pyrrhuloxia	Male	USNM 642143	CA 0.57; αD 0.13; AD 0.11; EC 0.1; AS 0.07; LU 0.02
Carduelis chloris	European goldfinch	Male	USNM 637389	XB 0.54; XC 0.32; XA 0.09; LU 0.05
Coereba flaveola	Bananaquit	Male	USNM 639172	ZE 0.59; LU 0.21; AH 0.2
Colaptes auratus	Northern flicker	Female	USNM 623435	LU 0.6; ZE 0.2; PI 0.1; DH 0.1;
Cotinga cotinga	Purple-breasted cotinga	Male	USNM 632564	CO 0.8; CA 0.2
Emberiza melanocephala	Black-headed bunting	Female	USNM 637386	LU 0.8; DH 0.2
Euphonia laniirostris	Thick-billed euphonia	Male	USNM 643899	LU 0.64; CL 0.14; DH 0.12; ZE 0.08; AH 0.02
Euphonia saturata	Orange-crowned euphonia	Male	USNM 643992	LU 0.4; ZE 0.3 CL 0.1; DH 0.1; AH 0.1
Icterus galbula	Baltimore oriole	Male	USNM 623444	LU 0.36; XB 0.29; XC 0.15; XA 0.09; CA 0.06; DH 0.05
Icterus icterus	Venezuelan troupial	Male	USNM 632598	LU 0.3; XA 0.2; XC 0.2; ZE 0.2; XB 0.1
Melanerpes formicivorus	Acorn woodpecker	Male	USNM 641593	αD 0.9; AD 0.1
Oreothlypis ruficapilla	Nashville warbler	Male	USNM 637605	LU 0.7; CL 0.2; DH 0.1
Paroaria coronata	Red-crested cardinal	Female	USNM 643469	αD 0.39; CA 0.28; AD 0.15; AS 0.08; EC 0.06 LU 0.04;
Phaethon rubricauda	Red-tailed tropicbird	Male	USNM 632100	αD 0.65; AS 0.28; CA 0.07
Phoeniconaias minor	Lesser flamingo	Male	USNM 634731	CA 0.4; αD 0.2; AS 0.2; AD 0.1; EC 0.1
Picoides villosus	Hairy woodpecker	Male	USNM 639056	αD 1
Picumnus exilis	Golden-spangled piculet	Male	USNM 639369	αD 0.5; LU 0.3; AD 0.2
Piranga flava	Red tanager	Male	USNM 643860	XC 0.5; XB 0.4; XA 0.1
Piranga ludoviciana	Western tanager	Male	USNM 634993	XC 0.5; XB 0.4; XA 0.1
Platalea ajaja	Roseate spoonbill	Male	USNM 635736	αD 0.3; AS 0.2; CA 0.2; AD 0.2; EC 0.1
Ploceus velatus	Southern masked weaver	Male	USNM 642356	LU 0.8; ZE 0.2
Pteroglossus aracari	Black-necked ara□ari	Female	USNM 637112	αD 0.74; AD 0.16; LU 0.08; CA 0.02
Pyrrhula pyrrhula	Eurasian bullfinch	Male	USNM 637523	αD 0.6; AS 0.4
Ramphastos tucanus	White-throated toucan	Male	USNM 632532	αD 0.7; AD 0.1; XC 0.1; LU 0.1
Selenidera piperivora	Guianan toucanet	Male	USNM 632544	αD 0.86; LU 0.08; AD 0.03; CA 0.03
Serinus mozambicus	Yellow-fronted canary	Male	USNM 636670	XC 0.66; XA 0.18; XB 0.16;
Setophaga petechia	American yellow warbler	Unknown	USNM 638043	LU 0.69; DH 0.17; ZE 0.09; AH 0.05
Sicalis flaveola	Saffron finch	Female	USNM 635754	LU 0.5; ZE 0.2; CLE 0.12; DH 0.11; AH 0.07
Telophorus zeylonus	Bokmakierie	Female	USNM 642574	XC 0.73; XA 0.24; XB 0.03
Trogon mesurus	Ecuadorian trogon	Male	USNM 643987	CA 0.66; AD 0.18; EC 0.07; αD 0.05; AS 0.03; HE 0.01;
Tyrannus vociferans	Cassin's kingbird	Male	USNM 642152	LU 0.4; αD 0.4; ZE 0.2
Vestiaria coccinea	'I'iwi	Unknown	USNM 634051	αD 0.39; CA 0.31; AS 0.13; AD 0.12; EC 0.05
Zosterops japonicus	Japanese white-eye	Female	USNM 641812	LU 1

[a]Results from HPLC: αD, α-doradexanthin; AD, adonirubin; AH, anhydrolutein; AS, astaxanthin; CA, canthaxanthin; CL, cis-isomer of lutein; CO, cotingin; DH, dehydrolutein; EC, echinenone; HE, 3-hydroxy-echinenone; LU, lutein; PI, 'picofulvin; XA, canary xanthophyll a; XB, canary xanthophyll b; XC, canary xanthophyll c; ZE, zeaxanthin.
[b]Specimens from the National Museum of Natural History, Smithsonian Institution, Washington, DC, USA.

and the triplicate spectra from each feather were averaged. The matrix of intensity values was mean-centered.[22] Preprocessing and subsequent principal component analysis (PCA) of the spectral intensity values was performed in R 2.15.2:[24] PCA was performed with the 'prcomp' function.

HPLC spectra from all 36 feathers were analysed with Empower 5.0 software (Waters Corporation, Milford, MA, USA). A baseline was manually fitted to two-dimensional spectra from the 441, 448, 454, 468 and 476 nm channels and absorption peaks were delimited. Peak area (absorption units) was recorded for peaks from the channel nearest to their λ_{max}. Carotenoids were identified by comparison with the retention time (t_R) and absorption maxima (λ_{max}) of standards that had previously been analysed on this system (Table 2). The relative abundance of each carotenoid in each feather was calculated from the fraction of carotenoid peak areas.

2.4 Results and Discussion

The most abundant pigment in each of the studied feathers was one of six carotenoids: canthaxanthin, canary xanthophylls (mixture of A, B, and C), cotingin, α-doradexanthin, lutein or zeaxanthin.[†] Other pigments extracted from feathers included adonirubin, anhydrolutein, astaxanthin, a cis-isomer of lutein, dehydrolutein, echinenone, 3-hydroxy-echinenone and 'picofulvin' (Table 1). All spectra featured 'carotenoid' bands at 1500–1535 cm^{-1} (identified as ν[C=C]), 1145–1165 cm^{-1} (identified as ν[C–C]) and 1000–1010 cm^{-1} (identified as δ[CH_2]), and bands attributed to β-keratin were absent or minor.[12,22] The most intense band from β-keratin was removed during spectral preprocessing (1420–1485 cm^{-1}). Small bands that characterise functional groups in specific carotenoids were variably present between 1165 and 1500 cm^{-1}, including a set of three bands at 1260, 1280 and 1294 cm^{-1} in spectra from purple feathers (purple-breasted cotinga, *Cotinga cotinga*).

The width and position of the three major 'carotenoid' bands, and the presence of 'carotenoid-specific' bands between 1165 and 1500 cm^{-1}, were major sources of variation within the spectral dataset. The first four components from the PCA

Table 2 Reference HPLC parameters for identifying carotenoids.[a]

Carotenoid	Retention time (tR, min)	Absorption (λ_{max}, nm)
Adonirubin	7.2	482
Anhydrolutein	8.9	448
Astaxanthin	6.8	479
Canary xanthophyll A	5.4	439
Canary xanthophyll B	6.0	444
Canary xanthophyll C	4.7	438
Canthaxanthin	8.5	471
Cotingin	7.3	476
Dehydrolutein	5.8	448
α-Doradexanthin	5.5	473
Echinenone	9.9	467
3-Hydroxy-echinenone	11.9	466
Lutein	6.3	448
Lutein (cis-isomer)	5.3	441
'Picofulvin'	8.0	441
Zeaxanthin	7.3	473

[a]From standards previously run on the same system as the pigments studied here, except for cotingin, which was inferred with respect to Mendes-Pinto et al.[15]

explained 96.4% of the variation (71.7, 14.4, 6.6 and 3.7% for principal component one (PC1), PC2, PC3 and PC4, respectively). Principal component one was heavily influenced by ν[C=C] position, and PC4 was mostly influenced by ν[C–C] position (Fig. 1). In contrast, PC2 and PC3 were strongly influenced by the relative intensities of ν[C=C] and ν[C–C].

Principal component analysis reduces each Raman spectrum to a single 'score' for each PC, where similar spectra (with respect to each PC) will have similar PC scores.[24] In our analysis, we found that the spectra with similar PC1 and PC4 scores had similar carotenoid compositions. The averaged spectra from the purple feather of a purple-breasted cotinga had the highest PC1 score. The primary feather pigment in the purple feather was a methoxy-keto-carotenoid (cotingin).[11] Spectra with canthaxanthin as the primary feather pigment also had high PC1 scores. The PC1 scores of feathers with α-doradexanthin as the primary feather pigment ranged from positive through to negative. Raman spectra from piciform feathers (i.e. an ara□ari, a piculet, a toucan and two woodpeckers) had the most negative PC1 scores of all feathers rich with α-doradexanthin. Spectra from α-doradexanthin-rich feathers and spectra from feathers rich with yellow carotenoids had overlapping PC1 scores; however, the α-doradexanthin-rich spectra and yellow carotenoid-rich spectra had different PC4 scores. Spectra from feathers with lutein as the primary pigment had negative PC1 scores. The red feather from Cassin's kingbird (*Tyrannus vociferns*) had similar proportions of α-doradexanthin and lutein and had PC1 and PC4 scores similar to spectra from lutein-rich feathers. Spectra from feathers rich with canary xanthophylls typically had the lowest PC1 scores: key exceptions include the lutein-rich feather of a Japanese white eye (*Zosterops japonica*) and canary xanthophyll-rich feather of a Western tanager (*Piranga ludoviciana*) (Fig 1). Zeaxanthin was the primary pigment in the feather from a bananaquit (*Coereba flaveola*) and spectra had PC1 and PC4 scores similar to lutein-rich feathers.

Principal component one described the effective conjugation length of carotenoid feather pigments. The purple feather with cotingin as the major pigment had the highest PC1 score: the conjugated system of cotingin has 11 conjugated subunits (C=C–C) in the backbone, which are cross-conjugated with subunits in the beta rings (conjugation is bridged by keto groups in the 4 and 4' positions in the beta rings).[11] The slightly lower PC1 scores from canthaxanthin are attributed to a slightly shorter effective conjugation length, where 11 conjugated subunits in the backbone are continuous with 4 and 4' keto groups in the beta rings (i.e. no cross-conjugation).[26] Conjugation in α-doradexanthin includes nine conjugated subunits in the backbone continued by a 4 keto group in one beta ring; PC1 scores for α- doradexanthin were lower than those for canthaxanthin.[27] The conjugated bond system of zeaxanthin includes 11 conjugated subunits, with conjugation extending into each of the beta rings.[28] The mean PC1 score for the zeaxanthin-pigmented bananaquit feather was less than that for the red feather pigments. The conjugated system of lutein includes 10 conjugated subunits, with conjugation extending into only one of the beta rings;[29] the mean PC1 score representing lutein-rich feathers was less than the PC1 score for the averaged bananaquit spectra. Finally, the conjugated systems for canary xanthophylls A, B and C are limited to nine conjugated subunits in the backbone.[30] Spectra from feathers rich with canary xanthophylls had the lowest mean PC1 score.

Spectra and consequent PC1 scores were likely influenced by secondary carotenoids in addition to the primary feather pigment; describing secondary carotenoids might be possible with a larger dataset.

2.5 Method Test

A PC score for any new sample can be calculated as the dot product of a loadings vector multiplied by a Raman spectrum. A dot product is calculated in three steps:[24] 1) equal length vectors are aligned (i.e. new Raman spectrum and loadings vector), 2) corresponding entries in each vector are multiplied, and 3) the sum of all products is calculated. Dot products calculated using PC1 and PC4 loadings vectors were treated as the PC1 and PC4 scores for the new spectrum, and could be projected amongst the scores of existing spectra. The major carotenoid represented by the new spectrum could then be determined from existing spectra by finding the shortest Euclidean distance between the new and existing scores. We had access to an additional feather from the purple-breasted cotinga, roseate spoonbill (*Platalea ajaja*) and white-throated toucan (*Ramphastos tucanus*). We collected triplicate Raman spectra from each of these additional feathers to test the dot product method. Principal component one and PC4 dot products (i.e. scores) were calculated for the new spectra and Euclidean distances to existing [PC1, PC4] coordinates were determined. The shortest Euclidean distance for each of the new spectra were to existing spectra from the same individual (i.e. the PC1 and PC4 scores for the new purple-breasted cotinga spectra were most similar to the PC1 and PC4 scores from existing purple-breasted cotinga spectra).[†]

One feather from seven additional species was studied with Raman spectroscopy (Table S1).[†] Feather carotenoids from each of the seven species had previously been studied with HPLC (different individuals to those studied here).[4,5,31-34] The

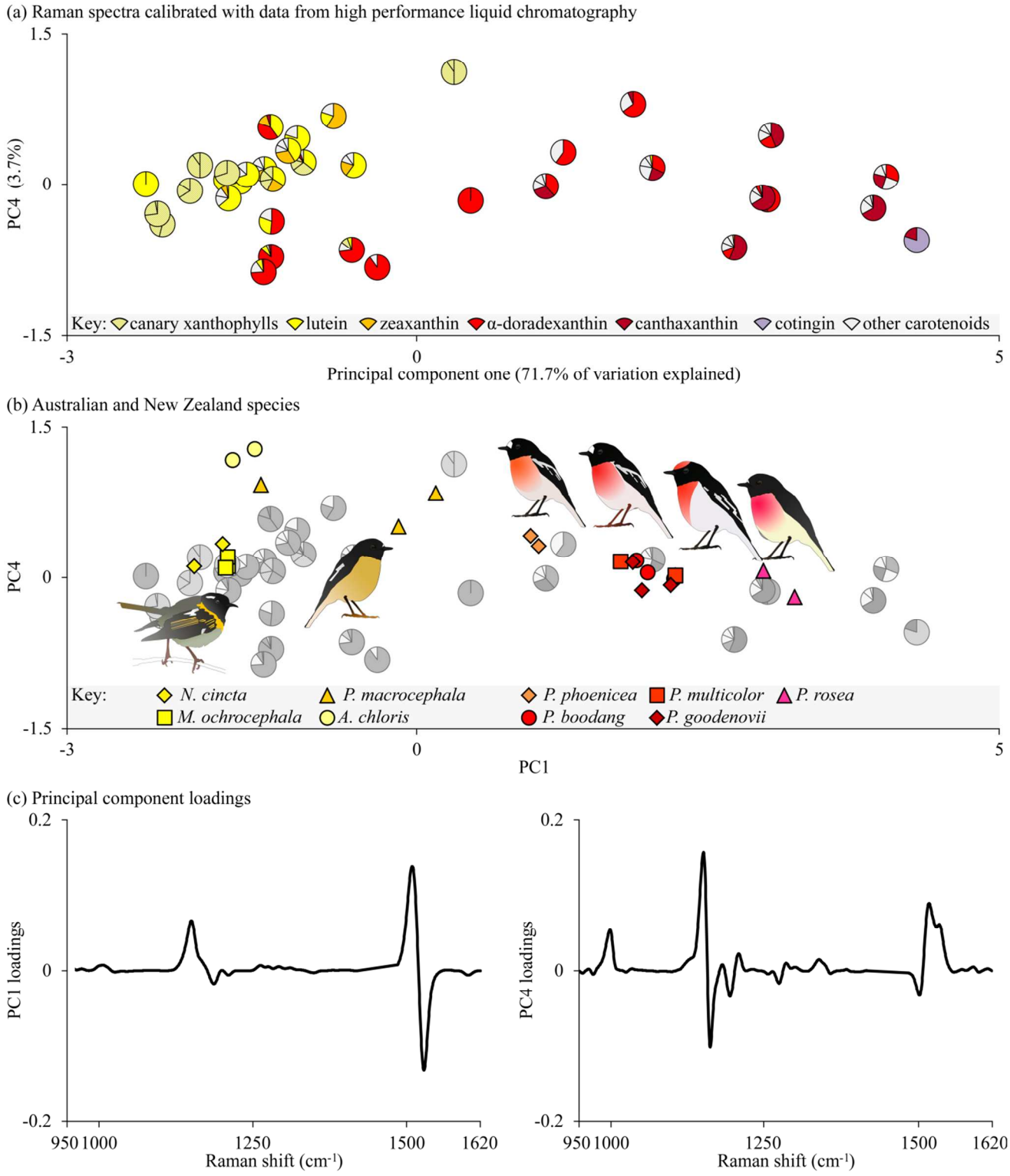

Fig. 1 (a) Principal component one (PC1) and principal component four (PC4) scores for mean Raman spectra from 36 feathers. Scores are represented by pie charts[35] showing the proportions of carotenoids subsequently extracted from the feather (Table 1). (b) Raman spectra from the feathers of Australian and New Zealand birds. Red or orange feathers on several Australian species are predicted by Raman spectroscopy and principal components analysis to be pigmented with α-doradexanthin. (c) Loadings for PC1 and PC4 are useful for interpreting variation between spectra. The Raman spectral bands with both positive and negative weighting are the bands that varied with carotenoid composition.

major carotenoids predicted for six of these seven feathers were consistent with previous studies (*Euplectes capensis* — lutein; *Fringilla montifringilla* — lutein; *Parus major* — lutein; *Ploceus bicolor* — lutein; *Regulus regulus* — lutein; *Uragus sibiricus* — α-doradexanthin). Raman spectra from an orange wing feather of a Red-billed Leiothrix (*Leiothrix lutea*) were here predicted to contain abundant zeaxanthin, which is inconsistent with a previous report of lutein and dehydrolutein from a yellow *Leiothrix lutea* feather.[33]

3 Method Application

3.1 Background

Raman spectroscopy is useful for studying plumage carotenoids in museum specimens, where feather colours might provide insight into the evolution and ecology of birds but destructive sampling of feathers is difficult to justify. Specimens that are rare, old or significant for other reasons may be better suited for non-destructive analysis of feather pigmentation. Here we have collected Raman spectra from 23 specimens housed in the National Museum of Natural History, Smithsonian Institution, that were acquired between 1872 and 1938.† These old and rare specimens include endangered species and were studied for insight into the evolution of plumage colouration in New Zealand birds.

Red carotenoids are relatively common feather pigments in songbirds across the world but are apparently absent from the feathers of endemic New Zealand species. The trend away from bright pigmentation and towards muted and cryptic feather-patterning is most keenly observed among *Petroica* robins: species are orange, red or magenta in Australia, New Guinea and smaller Pacific Islands, and light yellow, grey or black in New Zealand.[36] A colour shift by New Zealand *Petroica* species, coupled with the apparent absence of red carotenoids in the feathers of other New Zealand birds, hints at a bias against red-pigmented feathers. One explanation for the restricted plumage palette of New Zealand may be a selection pressure against large displays of red feathers. Mechanistically, such a selection pressure may work against the metabolic conversion of yellow dietary carotenoids (i.e. lutein) into red keto-carotenoids (e.g. α-doradexanthin).[5] An endemic New Zealand species with lutein-rich plumage that had an ancestor with α-doradexanthin-rich plumage would be preliminary evidence for a 'colour shifting' selection pressure. Accordingly, we can predict whether a colour shift has occurred with an ancestral state reconstruction.† Here we analyse carotenoids in the colorful plumages of Australian and New Zealand *Petroica* species, as well three other New Zealand species, to seek evidence of a 'colour shifting' selection pressure.

3.2 Materials and Methods

Pigments were studied in 69 feathers from 23 individual study skins representing nine species (Table S1)† Thirteen specimens from Australia were studied including three male flame robins

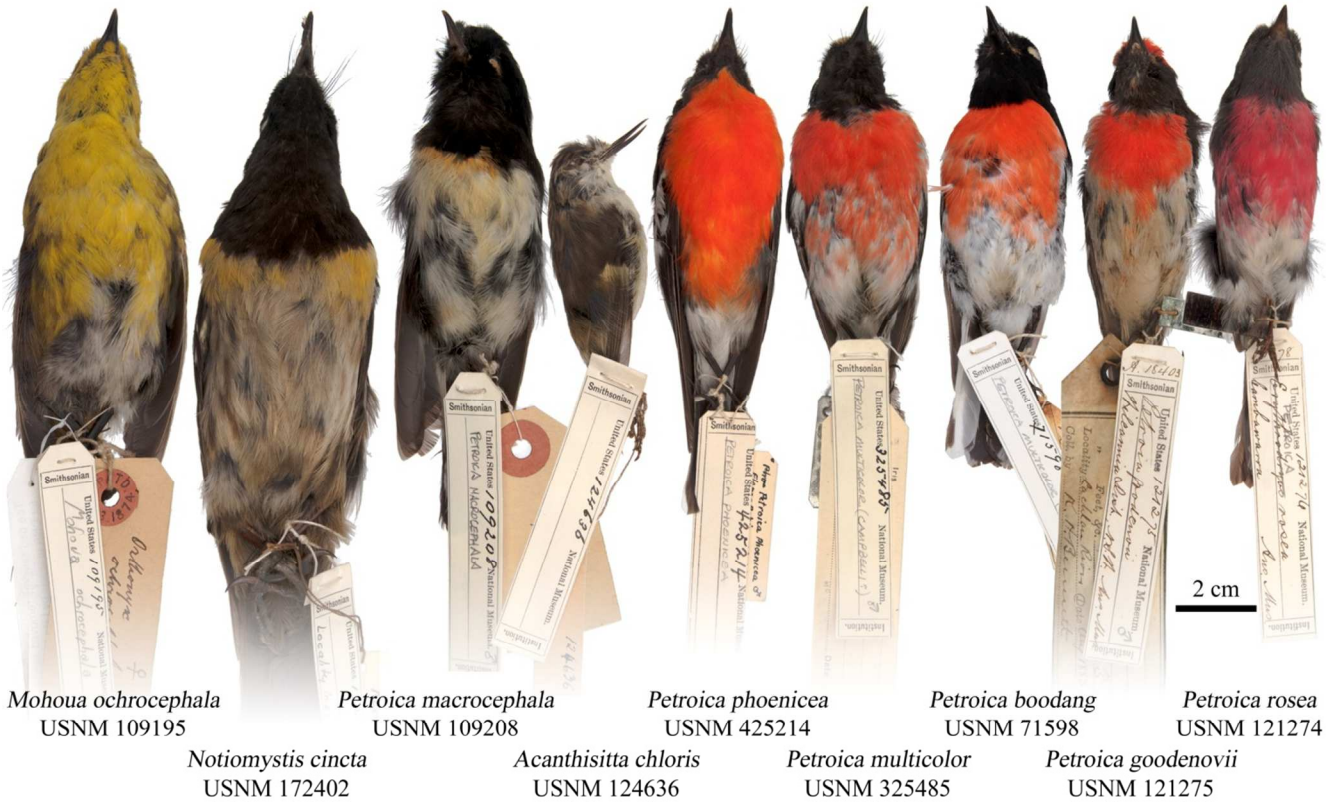

Fig. 2 A selection of the New Zealand and Australian birds that were analysed. Birds from New Zealand include mohua (Mohoua ochrocephala), hihi (Notiomystis cincta), miromiro (Petroica macrocephala) and tītipounamu (Acanthositta chloris). Australian species include flame robin (P. phoenicea), Pacific robin (P. multicolor), scarlet robin (P. boodang), red-capped robin (P. goodenovii) and rose robin (P. rosea).

(*Petroica phoenicea*; orange feathers), three male red-capped robins (*P. goodenovii*; red feathers), three male scarlet robins (*P. boodang*; red feathers), two male Pacific robins (*P. multicolor*; red feathers) and two male rose robins (*P. rosea*; magenta feathers)(Fig. 2). The New Zealand avifauna was represented by three male miromiro (*P. macrocephala*; yellow feathers), two male and one female mohua (*Mohoua ochrocephala*; yellow feathers), two male hihi (*Notiomystis cincta*; yellow feathers) and one male and one female tītipounamu (*Acanthositta chloris*; yellow feathers)(Fig. 2).

Three Raman spectra were collected from each study skin; each spectrum was collected from a different feather. Analyses were performed using the previously described instrument and settings. Entire study skins were placed beneath the microscope objective for data collection (i.e. feathers were not plucked)

3.3 Results and Discussion

The flame, Pacific, red-capped and scarlet robins were predicted by Raman spectroscopy and principal components analysis to be pigmented with α-doradexanthin (Fig. 1). Spectra from the feathers of these four *Petroica* species were most similar to spectra from the red-crested cardinal (*Paroaria coronata*) feather.† Spectra from the rose robin feathers were most similar to spectra from the northern cardinal (*Cardinalis cardinalis*) feather, and were predicted to have canthaxanthin as the major carotenoid.

Passerines from New Zealand may contain dietary carotenoids and metabolically derived pigments. The mohua feathers were predicted to contain canary xanthophylls. This result is ambiguous, as PC1 and PC4 scores of the mohua spectra were similar to the scores from both the cedar waxwing (*Bombycilla cedrorum*; canary xanthophylls) and southern masked weaver (*Ploceus velatus*; lutein) spectra. Spectral signals from hihi and tītipounamu feathers, both of which are weakly yellow and dusky coloured, were too low to make accurate predictions. For example, hihi were here predicted to display canary xanthophylls, which is inconsistent with previous HPLC results.[36] Spectra from tītipounamu produced PC1 and PC4 scores that were substantially different from the scores of HPLC-calibrated spectra. Spectra from hihi and tītipounamu feathers usefully demonstrate the influence of carotenoid composition and the importance of collecting high-quality spectra. Here we find that a high-quality spectrum can be distinguished by a baseline corrected ν[C=C] carotenoid band that is more than 20 times taller than the baseline corrected ν[C–H] keratin band around 2950 cm^{-1}. Each spectrum from the southern masked weaver feather had a carotenoid:keratin ratio of 29 or greater and produced PC1 and PC4 scores that were similar to other spectra from lutein rich feathers. In contrast, spectra from hihi and tītipounamu feathers had carotenoid:keratin ratios that were typically less than 10 and provided spurious results. Spectra with relatively weak pigment bands may not contain enough information for correct pigment identification, and may result from low concentrations of carotenoids or co-deposition of fluorescent melanins in feathers.

Flame, Pacific, red-capped and scarlet robins in Australia are predicted to have plumages rich with α-doradexanthin, which can be a metabolic derivative of lutein.[5] Lutein is used as a plumage pigment by New Zealand birds,[37] and may be displayed by miromiro. These are two alternative evolutionary scenarios that may explain these plumage pigment differences: 1) the metabolic conversion of α-doradexanthin may have been evolutionarily lost in *Petroica* robins from New Zealand, or 2) metabolic conversion of lutein to α-doradexanthin may have evolved relatively recently in *Petroica* species from Australia. The former scenario is predicted from an ancestral state reconstruction,† and therefore, we propose that the plumage of *Petroica* migrants to New Zealand shifted from red to yellow. New Zealand and Australian *Petroica* are a good study system to understand gains and losses of metabolically-altered carotenoid displays.

4 Conclusions

Non-destructive Raman spectroscopy can be paired with principal component analysis to non-invasively identify the most abundant carotenoid in colourful bird feathers. The method is most effective with feathers that are strongly coloured and when carotenoids are not co-deposited with melanins in feathers. The subtle spectral variations that identify each carotenoid are attributed to differences in effective conjugation lengths of the carotenoid molecules. A statistical model for discriminating Raman spectral properties of feather carotenoids was effective at predicting types of carotenoid pigmentation, i.e. the red plumage of a white-throated toucan was spectrally and chemically distinct from the red plumage of a northern cardinal. We collected Raman spectra from museum specimens that were up to 140 years old without plucking feathers, and this provided insight into the evolution of plumage colours in an island lineage of songbirds. Additional HPLC-calibrated Raman spectra would extend the list of carotenoids that might be identified in feathers with non-destructive Raman spectroscopy.

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†*Electronic Supplementary Information (ESI) available: Specimens analysed in the current study (Table S1); Description of ancestral state reconstruction for Petroicidae; Petroicidae phylogeny with plumage characters coded (Fig. S1).* See DOI: 10.1039/b000000x/

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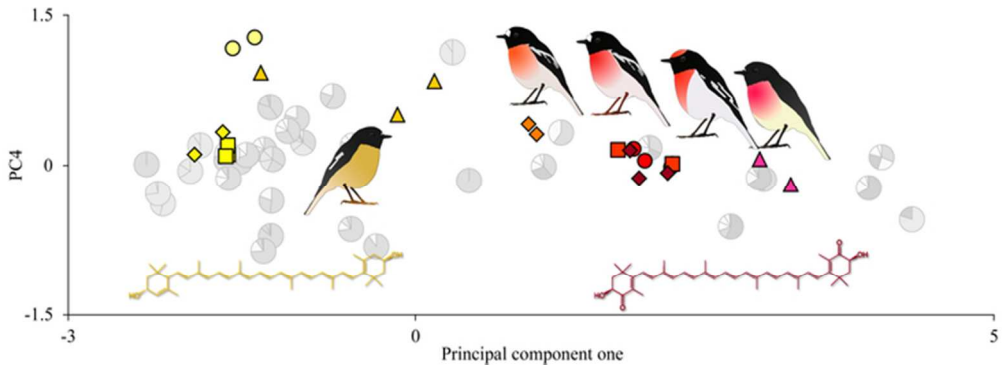

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