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RESEARCH ARTICLE

Nondestructive Raman spectroscopy confirms carotenoid-pigmented plumage in the Pink-headed Duck

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ABSTRACT

A small group of pigment classes is responsible for the wide range of plumage colors in modern birds. Yellow, pink, and other "warm" feather colors of many species are attributed to carotenoid pigments, a plumage trait that has an uneven distribution across modern bird species. Carotenoid plumage pigments are especially rare among fowl (superorder Galloanseres), and until recently, the Pink-eared Duck (*Malacorhynchus membranaceus*) from Australia provided the only evidence that any species of waterfowl (order Anseriformes) exhibits carotenoid-pigmented plumage. We analyzed a Pink-headed Duck (*Rhodonessa caryophyllacea*) study skin using Raman spectroscopy, without plucking or otherwise damaging the specimen. Raman spectra confirmed that the pink feathers of *Rhodonessa* are pigmented with carotenoids. Spectra from *Rhodonessa* were similar to those from *Malacorhynchus*, which suggests that the same carotenoid is the primary plumage pigment in both species. Moreover, spectra from *Rhodonessa* were similar to spectra from other taxa pigmented with ketocarotenoids. *Malacorhynchus* and *Rhodonessa* are distant relatives within Anseriformes, so these findings indicate multiple evolutionary origins of plumage carotenoids within the waterfowl or (less likely) many losses of plumage carotenoids from duck species. Our results show that pigment chemistry can be studied in precious ornithological specimens without damaging the specimens, and provide new evidence that the (apparently extinct) *Rhodonessa* possessed what is evolutionarily an extremely rare trait among waterfowl.

Keywords: Anseriformes, coloration, feather, pigmentation, Raman spectroscopy, *Rhodonessa*

La espectrometría Raman no destructiva confirma la pigmentación con carotenoides del plumaje de *Malacorhynchus membranaceus*

RESUMEN

Un pequeño grupo de clases de pigmentos es responsable del amplio rango de colores del plumaje en las aves modernas. Amarillo, rosa y otros colores "cálidos" de las plumas de muchas especies son atribuidos a los pigmentos carotenoides, un rasgo del plumaje que tiene una distribución desigual entre las especies de aves modernas. Los pigmentos carotenoides del plumaje son especialmente raros entre las aves de caza (superorden Galloanseres) y hasta hace poco, la especie *Malacorhynchus membranaceus* de Australia representaba la única evidencia de una especie de ave acuática (orden Anseriformes) con plumaje pigmentado con carotenoides. Analizamos una piel de estudio de *Rhodonessa caryophyllacea* usando espectrometría Raman sin perforar o dañar el espécimen. El espectro Raman confirmó que las plumas rosas de *Rhodonessa* están pigmentadas con carotenoides. Los espectros de *Rhodonessa* fueron similares a aquellos de *Malacorhynchus*, sugiriendo que el mismo carotenoide es el principal pigmento del plumaje en cada especie. Más aun, los espectros de *Rhodonessa* fueron similares a los espectros de otros taxa pigmentados con ceto-carotenoides. *Malacorhynchus* y *Rhodonessa* son parientes distantes adentro de los Anseriformes, indicando orígenes evolutivos múltiples de los carotenoides del plumaje adentro de las aves acuáticas, o (menos probable) muchas pérdidas de los carotenoides del plumaje en las especies de patos. Nuestros análisis muestran que la química de los pigmentos puede ser estudiada en especímenes ornitológicos valiosos sin dañarlos, y brinda nueva evidencia de que la especie (aparentemente extinta) *Rhodonessa* poseía lo que es un rasgo evolutivo extremadamente raro entre las aves acuáticas.

Palabras clave: Anseriformes, coloración, espectrometría Raman, pigmentación, plumas, *Rhodonessa*

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INTRODUCTION

Mechanisms for accumulating at least 6 chemically distinct groups of pigments in feathers have evolved within birds or their nonavian ancestors (McGraw 2006b, Thomas et al. 2013). Carotenoid plumage pigments (red, orange, yellow, pink, purple) are honest signals of fitness for some species (Hill 1990) and apparently have multiple, ancient origins (Thomas et al. 2014a). Curiously, species that display plumage carotenoids have an uneven phylogenetic distribution across the radiation of modern birds and are rare or absent among the most basal lineages: absent in ratites (Paleognathae) and rare in fowl (Galloanseres) (Thomas et al. 2014a).

The bill keratin and leg scales of several species of fowl are known to be pigmented with carotenoids (e.g., Red Junglefowl [*Gallus gallus*] and Upland Goose [*Chloephaga picta leucoptera*]; McGraw and Klasing 2006, Gladbach et al. 2010), and these traits likely have a broad taxonomic distribution across Galloanseres (for orange and red integuments, see del Hoyo et al. 1992; for a summary, see Olson and Owens 2005). By contrast, carotenoid pigments have been identified only in the plumages of 3 chicken-like bird species (*Chrysolophus pictus*, *Haematortyx sanguiniceps*, and *Ithaginis cruentus*; order Galliformes) and one species of waterfowl (order Anseriformes) (for a summary, see Thomas et al. 2014a). Specifically, in waterfowl, carotenoids are responsible for the "pink ears" of the Pink-eared Duck (*Malacorhynchus membranaceus*). On the basis of this isolated occurrence, the evolution of carotenoid feather pigmentation in *M. membranaceus* was predicted to have been a novel event, convergent with other displays of plumage carotenoids (Thomas et al. 2014a). However, the evolutionary interpretation for carotenoid plumage pigments in waterfowl may be biased by living taxa. The apparently extinct Pink-headed Duck (*Rhodonessa caryophyllacea*) also had pink-hued feathers.

Adult *Rhodonessa* were similar in size to Mallards (*Anas platyrhynchos*) and had a mostly dark brown plumage, with the exception of white regions on the wings and pink feathers on the neck and head (Baker 1908). Males and females were visually distinguishable during the breeding season by the high saturation of pink in the feathers of males (Baker 1908). The endemic range of *Rhodonessa* included wetlands in Bangladesh, India, Nepal, and Myanmar. *Rhodonessa* was last sighted in India in 1949 and has been considered critically endangered since 1994 (Tordoff et al. 2008, Birdlife International 2012). Recent expeditions into central and northern Myanmar sought *Rhodonessa* without success (Eames 2008, Tordoff et al. 2008). *Malacorhynchus* and *Rhodonessa* were distant relatives within Anseriformes, the former being more closely allied with stiff-tailed ducks (*Oxyura* spp.) and the latter more closely related to pochards (*Netta* spp.) (Livezey 1996, Worthy 2009).

The pink plumage of *Rhodonessa* is a striking trait for which biochemical data have not been reported. The pink coloration in the feathers of other species has been chemically identified as carotenoid pigmentation (e.g., American Flamingo [*Phoenicopterus ruber*] and Painted Stork [*Mycteria leucocephala*]; Fox 1955, Thomas et al. 2014a). The pink pigment in the feathers of both *Malacorhynchus* and *Rhodonessa* may be a carotenoid (Auber 1957), in which case, further information about the particular type of carotenoid might provide deeper evolutionary insight. More than 40 types of carotenoid are known from the plumages of modern birds (reviewed in McGraw 2006a), and there is growing evidence for taxonomically meaningful patterns in carotenoid composition. Examples include the methoxy-ketocarotenoid "contingin," known only from the plumages of cotingas (Cotingidae); and "picofulvin" carotenoids, known only from the plumages of woodpeckers (Picinae) (Stradi et al. 1998, Mendes-Pinto et al. 2012). These pigments are produced through the metabolism of ingested carotenoids, and the taxonomic distributions of each pigment likely indicate a shared ancestry for the respective metabolic pathways. For the pink plumages of the 2 duck species, identifying a common metabolic carotenoid in each species could indicate a shared ancestry for their plumage pigmentation trait. Robust identifications of particular carotenoids currently require destructive chromatographic techniques; however, nondestructive Raman spectroscopy can provide some resolution of carotenoid type (Thomas et al. 2014b). For the present study, we considered the *Rhodonessa* study skin too valuable for destructive sampling and instead sought cursory information about pigment type only from Raman spectroscopy.

The occurrence of carotenoid feather pigments in both *Rhodonessa* and *Malacorhynchus* would present 2 evolutionary scenarios for this trait: (1) 2 independent evolutionary origins for the deposition of carotenoid pigments in feathers within Anseriformes (see Arendt and Reznick 2008) or (2) a single evolutionary origin with multiple trait losses. Scenario 1 may identify atavistic reappearances of the trait resulting from gene regulation (e.g., Romero et al. 2012) or evolution of separate biomolecular pathways between carotenoid absorption and display. Complex patterns of trait evolution have previously been observed in the waterfowl, including multiple losses of plumage dichromatism (Omland 1997) and multiple evolutionary origins of hemoglobin genes (McCracken et al. 2009). The evolutionary pattern describing pink pigmentation in waterfowl may be similarly complex. In particular, identical pigment compositions in the plumage of each duck species would be most consistent with an atavistic reappearance, or with multiple

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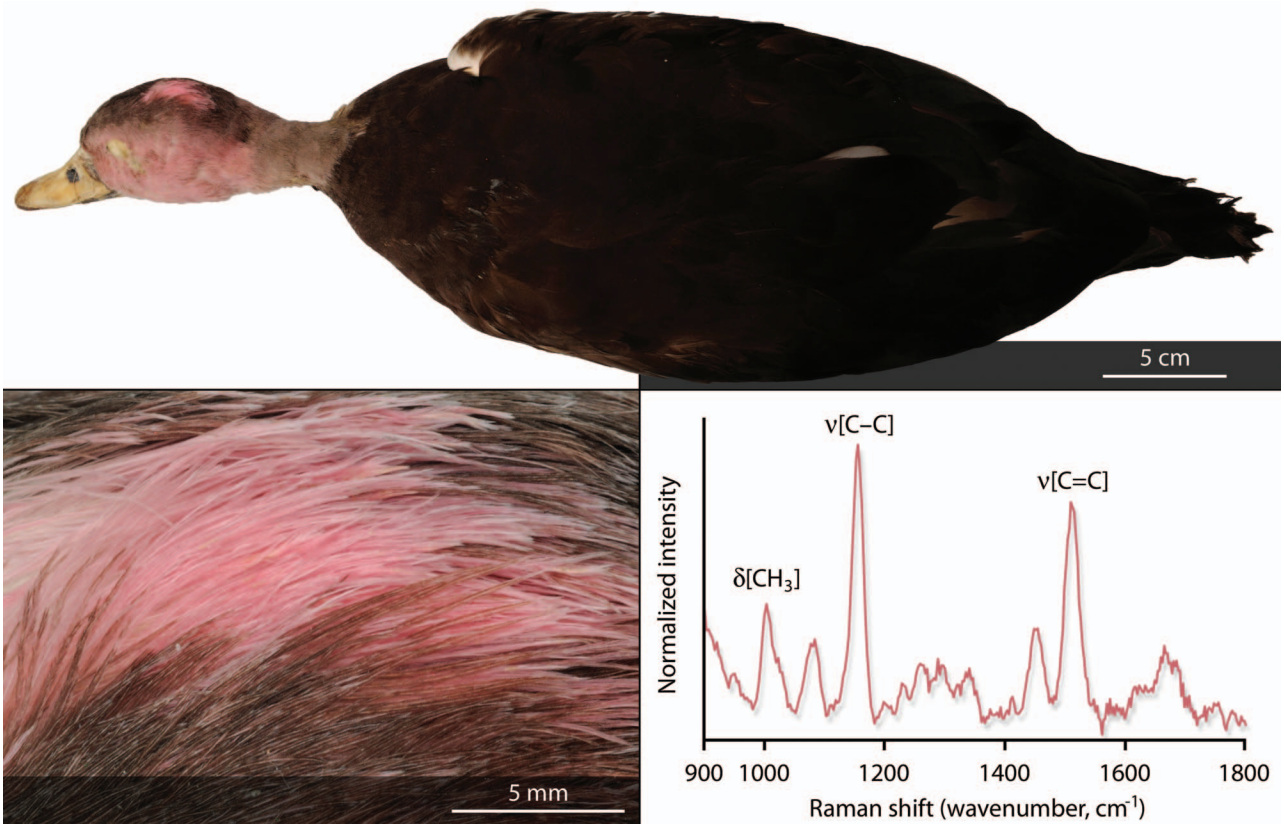

FIGURE 1. Dorsal view of a Pink-headed Duck (*Rhodonessa caryophyllacea*, USNM 608914), close view of the pink crown feathers, and a Raman spectrum collected from the pink feathers. The Raman spectrum was collected at 100 mW for 60 s through a 10× microscope objective. Each of the 3 carotenoid-identifying peaks in the spectrum has been labeled with the vibrational mode it represents (described in text).

trait losses in other duck taxa, but would not firmly reject independent evolutionary origins. Here, we primarily focus on phenotype description, with the aim of confirming carotenoid pigmentation in the pink feathers of *Rhodonessa*.

METHODS

Data Collection

The plumage of an adult male *R. caryophyllacea* (USNM 608914) was studied with Raman spectroscopy (Figure 1 and Supplemental Material Dataset S1). The specimen had been presented to the Smithsonian Institution National Museum of Natural History (NMNH, Washington, D.C., USA) on November 11, 1948, and had likely died in the aviary of Sir David Ezra and Lady Rachel Ezra (Calcutta, India). The bright pink feathers of the crown were studied with Raman spectroscopy and were not plucked or otherwise damaged. Eight Raman spectra were collected from the *Rhodonessa* study skin.

An earlier Raman spectroscopy study of plumage carotenoids had shown that the major type of carotenoid in a feather could be predicted from a Raman spectrum (Thomas et al. 2014b). Accordingly, *Rhodonessa* spectra were compared with spectra from feathers with known carotenoid compositions (60 spectra collected for this study; 20 different species, triplicate spectra from each feather; Table 1 and Supplemental Material Dataset S2). Likewise, *Malacorhynchus* spectra were compared with spectra from feathers with known carotenoid compositions (Supplemental Material Dataset S3; spectra from Thomas et al. 2014b). Feathers were from the consumptive feather file collection at the NMNH. Feathers were selected from the same individual birds that were studied in Thomas et al. (2014b).

The *Rhodonessa* study skin and comparative feathers were analyzed with a Nomadic Raman microscope (BaySpec, San Jose, California, USA), and both data collection and stage position were controlled with Spec 20/20 version 4.0.0.1 (BaySpec). Feathers were analyzed with an Nd:YAG laser (1,064 nm excitation; 500 mW maximum power), at 50 mW or 100 mW laser power, over 20 or 60 s. Data were collected using a 512 pixel InGaAs array detector with a spectral range of 277–1,886 cm^{-1}.

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TABLE 1. Raman spectra were collected from the feathers of 20 species to provide a comparative dataset for studying the major type of carotenoid present in the feathers of Pink-eared and Pink-headed ducks.

Common name	Scientific name	Collection ID
Lesser Flamingo	*Phoeniconaias minor*	USNM 634731
Roseate Spoonbill	*Platalea ajaja*	USNM 635736
Narina Trogon	*Apaloderma narina*	USNM 634596
Ecuadorian Trogon	*Trogon mesurus*	USNM 643987
Toco Toucan	*Ramphastos toco*	USNM 632532
Northern Flicker	*Colaptes auratus*	USNM 623435
Cassin's Kingbird	*Tyrannus vociferans*	USNM 642152
Purple-breasted Cotinga	*Cotinga cotinga*	USNM 632564
Bokmakierie	*Telophorus zeylonus*	USNM 642574
Cedar Waxwing	*Bombycilla cedrorum*	USNM 623482
Nashville Warbler	*Oreothlypis ruficapilla*	USNM 637605
Yellow Warbler	*Setophaga petechia*	USNM 638043
Bananaquit	*Coereba flaveola*	USNM 635754
Red-crested Cardinal	*Paroaria coronata*	USNM 643469
Western Tanager	*Piranga ludoviciana*	USNM 634993
Northern Cardinal	*Cardinalis cardinalis*	USNM 643555
Pyrrhuloxia	*Cardinalis sinuatus*	USNM 642143
Venezuelan Troupial	*Icterus icterus*	USNM 623444
Thick-billed Euphonia	*Euphonia laniirostris*	USNM 643899
'I'iwi	*Drepanis coccinea*	USNM 634051

The microscope used a 25 μm confocal pinhole and was equipped with an LMPlan N 10× IR microscope objective (Olympus, Melville, New York, USA) and an EPlan 40× microscope objective (Nikon Instruments, Tokyo, Japan); analysis spot sizes were ~4.3 μm and ~2 μm in diameter, respectively (Supplemental Material Dataset S1).

Spectra were processed with alternating point smoothing, Savitzky-Golay smoothing, and baseline correction in R 2.15.2 (R Development Core Team 2012). Alternating point smoothing was used to remove spectral noise introduced by the Nomadic Raman microscope. For alternating point smoothing, the spectrum of intensity values (IV) was divided into 2 vectors: one that included all the values with odd positions in the spectrum (odd vector $= IV_{1,3,5\ldots255}$) and a second that contained each other spectral value (even vector $= IV_{2,4,6\ldots256}$). The odd vector was normalized against the difference between the minimum and maximum intensity values, and this operation was repeated for the even vector. The 2 normalized vectors were then recombined into a single spectrum. The spectrum was then smoothed with a 5-point window using the "sgolay" function in the "signal" package (Signal Developers 2013). Finally, the smoothed spectrum was baseline-corrected using the "baseline" function in the "baseline" package (parameters were lambda = 1, hwi = 2, it = 10, int = 50, method = "fillPeaks"; Liland and Mevik 2012).

Spectrum Comparison

Previously published Raman spectra from Thomas et al. (2014a, 2014b) were also used for comparative study (Supplemental Material Dataset S3). In particular, spectra from *Malacorhynchus* plumage were compared to other spectra collected with 780 nm excitation. Spectra from *Rhodonessa* (here collected with 1,064 nm excitation) were compared to other spectra collected with 1,064 nm. Peak positions in selected spectra from each instrument (780 nm and 1,064 nm) were compared to confirm that both instruments were producing spectra with similar peak positions (Supplemental Material Table S1). Peak positions in *Malacorhynchus* and *Rhodonessa* spectra were subsequently compared for evidence of a shared carotenoid.

Raman spectra collected with 780 nm were analyzed with principal component (PC) analysis (following the method in Thomas et al. 2014b). The PC1 and PC4 scores were calculated for Raman spectra from the pink plumage of *Malacorhynchus*. The calculated score values were projected into PC1 and PC4 space. In a separate analysis, Raman spectra collected with 1,064 nm were analyzed with principal component analysis. The PC1 and PC4 scores were calculated for Raman spectra from the pink plumage of *Rhodonessa* and projected into PC space. Euclidean distance was calculated between the projected scores and each other score; small Euclidean distances were treated as evidence of similar carotenoid compositions (following Thomas et al. 2014b).

RESULTS

Evidence for Carotenoid Pigmentation

Raman spectra from the pink crown feathers of *Rhodonessa* have distinct peaks at 1,001, 1,075, 1,156, 1,447, 1,511, and 1,665 cm^{-1}, and a cluster of peaks between 1,225 and 1,325 cm^{-1}. The positions of the 3 tallest peaks are 1,156, 1,511, and 1,001 cm^{-1} (ordered in decreasing intensity after baseline correction; Figure 1), which is consistent with Raman spectra from carotenoids reported elsewhere (Veronelli et al. 1995, Thomas et al. 2014b). Accordingly, the 1,156, 1,511, and 1,001 cm^{-1} peaks are here attributed to the stretching of the single bonds in the carbon backbone (ν[C–C]), the stretching of double bonds in the carbon backbone (ν[C=C]), and the in-plane rocking mode of methyl groups attached to the carbon backbone (δ[CH_3]) (Veronelli et al. 1995). No peaks in spectra from *Rhodonessa* could be readily attributed to keto functionality (C = O; Berg et al. 2013). The apparent absence of a C = O peak may be a consequence of the relatively high noise in the spectra. Note that spectra from *Malacorhynchus* have a peak at 1,607 cm^{-1}, which may identify keto functionality.

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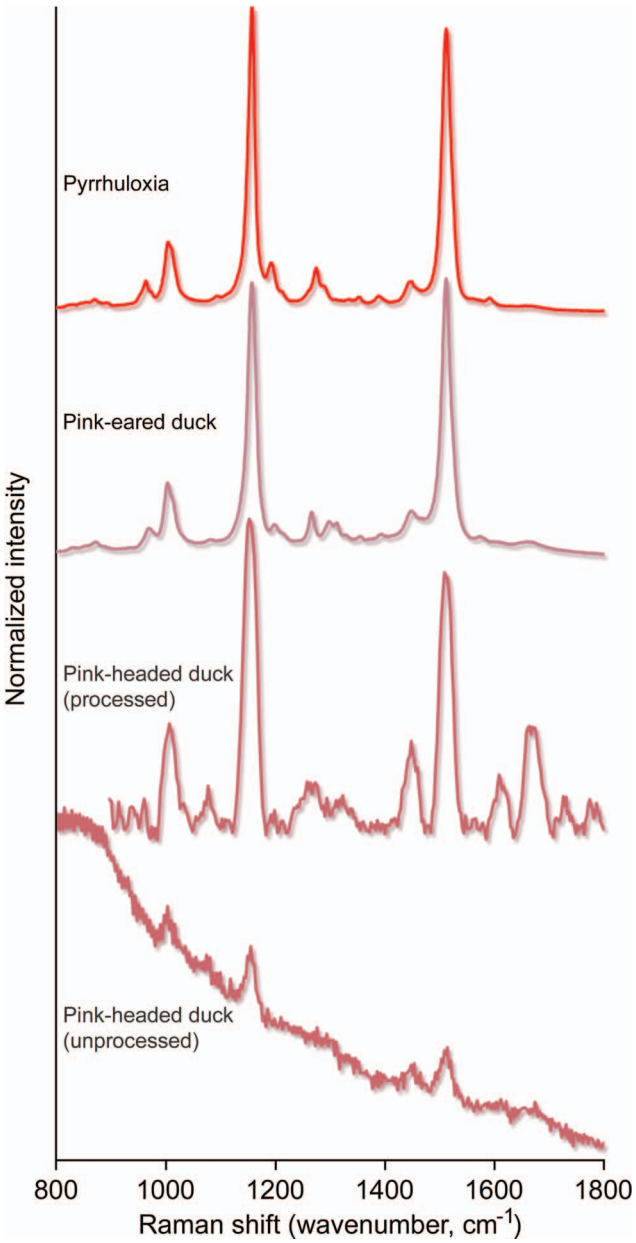

FIGURE 2. Raman spectrum from a Pyrrhuloxia (*Cardinalis sinuatus*) feather pigmented with canthaxanthin shown in comparison to spectra from pink duck feathers. Raman spectra from Pyrrhuloxia and Pink-eared Duck (*Malacorhynchus membranaceous*) were collected using 785 nm excitation. Raman spectra from Pink-headed Duck (*Rhodonessa caryophyllacea*) were collected using 1,064 nm excitation. Unprocessed and processed (baseline-corrected and smoothed) spectra from the Pink-headed Duck are shown. All spectra show peaks characteristic of carotenoids (see Figure 1).

The peaks at 1,225–1,325, 1,447, and 1,665 cm^{-1} are attributed to feather keratin, following previous assignments (Hsu et al. 1976). The Nomadic Raman microscope introduced a peak at 1,074 cm^{-1} into spectra from feathers that contain melanin, and it may identify an analytical artifact instead of a vibrational mode.

Spectrum Comparison

Spectra from the 2 instruments compare well: Wavenumber resolution of the 1,064 nm spectra was 2.5 cm^{-1}, and peak position differences for the 3 tallest peaks were ≤ 3 cm^{-1} (Figure 2 and Supplemental Material Table S1). The difference between *Rhodonessa* (1,064 nm instrument) and *Malacorhynchus* (780 nm instrument) spectra is also ≤ 3 cm^{-1} for identified peaks.

The PC scores calculated for *Malacorhynchus* feather spectra are similar to scores from Pyrrhuloxia (*Cardinalis sinuatus*) feather spectra (Supplemental Material Figure S1). Pyrrhuloxia feathers are primarily pigmented with the ketocarotenoid canthaxanthin (Thomas et al. 2014b). Scores calculated for *Rhodonessa* feather spectra are similar to scores from Lesser Flamingo (*Phoeniconaias minor*) feather spectra (Supplemental Material Figure S2). Lesser Flamingo feathers are primarily pigmented with the ketocarotenoid canthaxanthin (Thomas et al. 2014b). Canthaxanthin in Pyrrhuloxia and Lesser Flamingo feathers were previously identified by high-performance liquid chromatography (HPLC; Thomas et al. 2014b).

DISCUSSION

The pink feathers of *Rhodonessa caryophyllacea* are colored with carotenoid pigments. *Malacorhynchus membranaceus* and *R. caryophyllacea* are thus the only waterfowl species (order Anseriformes) with reported chemical evidence for carotenoid plumage pigments (Thomas et al. 2014a). The pink plumage in the 2 species may be pigmented with the same ketocarotenoid. Raman spectra from each of them have similar peak positions, indicating similar carotenoid compositions based on effective conjugation lengths (Veronelli et al. 1995; Supplemental Material Table S1). Scores calculated from the plumage of each of the 2 species were projected among canthaxanthin-pigmented feathers in PC space. This does not unquestionably confirm that the 2 ducks have identical pigment compositions, but it firmly supports that hypothesis. One goal of our study was to avoid feather destruction; unfortunately, feather plucking and destruction are required by the better-established analytical technique for carotenoid identification (i.e. HPLC). While HPLC methods of identifying plumage carotenoids have the benefit of multiple decades of method calibration and application (reviewed in McGraw 2006a), plumage carotenoid identification with nondestructive Raman spectroscopy is comparatively new, and our study indicates that it can be useful for plumage carotenoid analysis. Further calibration studies similar to Thomas et al. (2014b) should strengthen the inferences we can make from Raman

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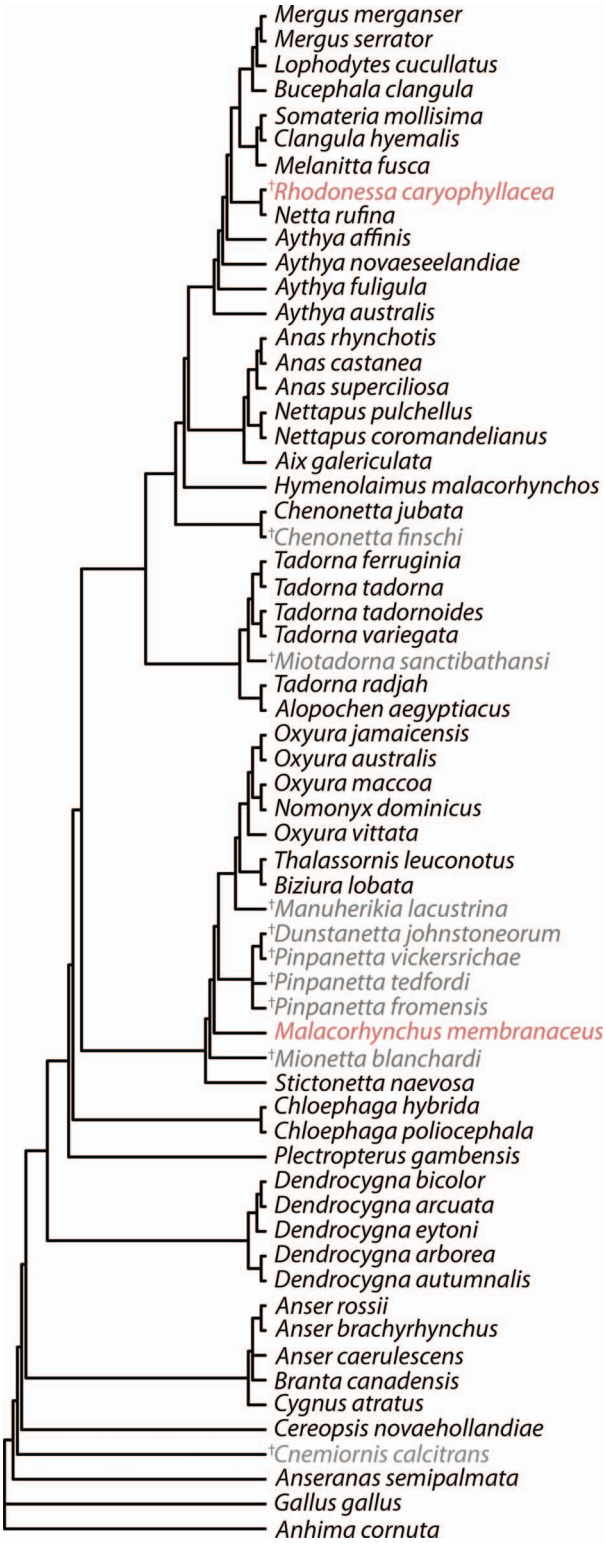

FIGURE 3. The Pink-headed Duck (*Rhodonessa caryophyllacea*) and Pink-eared Duck (*Malacorhynchus membranaceus*) are likely distant relatives within waterfowl (order Anseriformes). Carotenoid-pigmented plumage may have evolved twice within Anseriformes. To the best of our knowledge, these species have not been included in a single cladistic analysis; here, the phylogeny from Worthy (2009: figure 7) has been modified to show a sister relationship of *Rhodonessa* and *Netta* (Livezey 1996). Daggers denote extinct taxa. Species names are color coded to indicate presence or absence of carotenoids in plumage: pink = present, black = absent, and gray = unknown.

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spectra of plumage, so that in the future we can expect to identify the most abundant plumage carotenoid in the plumage of these 2 duck species without sample destruction.

A more precise evolutionary interpretation of this plumage trait will be possible once a well-resolved phylogeny of the Anatidae becomes available. What is clear is that the 2 species of waterfowl with carotenoid-pigmented plumage are not sister taxa and are probably distant from each other phylogenetically. *Rhodonessa* is allied with the tribe Aythyini (diving ducks), based on possession of an inflated and fenestrated syringeal bulla in males (Humphrey and Ripley 1962, Livezey 1996). Considering that the form of the syringeal bulla is a very reliable taxonomic character in Anseriformes (i.e. Sorenson et al. 1999), we do not doubt this assignment. Within Aythyini, *Rhodonessa* may be congeneric with *Netta* and sister to *Netta rufina* (Red-crested Pochard; Livezey 1996). The evolutionary relationships of *Malacorhynchus* appear to lie with the stiff-tailed ducks (Oxyurini; e.g., *Oxyura australis*; Worthy 2009). Modifying the anseriform phylogeny of Worthy (2009) to include *R. caryophyllacea* + *N. rufina*, we would regard *Malacorhynchus* and *Rhodonessa* as distant relatives within waterfowl (Figure 3). Independent appearances of plumage carotenoids in each lineage would have high statistical support, and the expression of carotenoid plumage pigments in each duck could conceivably result from convergent evolution.

Rhodonessa caryophyllacea is now the second species within Anseriformes and the sixth within Galloanseres known to have carotenoid plumage pigments (Thomas et al. 2014a). At present, the plumage carotenoid trait in *Rhodonessa* appears to have a novel evolutionary origin. Although the conservation priority of *Rhodonessa* would have been little influenced by its phylogenetic position (Jetz et al. 2014), the evolutionary significance of the plumage carotenoid trait might have brought positive attention to this duck if it had been discovered sooner. As it stands, the novelty of this plumage trait highlights the cost of extinction and the value of museum collections. One museum specimen of *Rhodonessa* has expanded our knowledge of plumage displays in Anseriformes, and reminded us of the sensitivity of evolutionary reconstructions to missing taxa.

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Author contributions: D.B.T. and H.F.J. conceived the experiment, wrote the paper, and analyzed the data. D.B.T performed the experiments and designed the methods. H.J.F. contributed materials and secured funding.

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