**RESEARCH ARTICLE**

**Impairment of mixed melanin-based pigmentation in parrots**

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**ABSTRACT**

Parrots and allies (Order Psittaciformes) have evolved an exclusive capacity to synthesize polyene pigments called psittacofulvin at feather follicles, which allows them to produce a striking diversity of pigmentation phenotypes. Melanins are polymers constituting the most abundant pigments in animals, and the sulphurated form (pheomelanin) produces colors that are similar to those produced by psittacofulvin. However, the differential contribution of these pigments to psittaciform phenotypic diversity has not been investigated. Given the color redundancy, and physiological limitations associated with pheomelanin synthesis, we hypothesized that the latter would be avoided by psittaciform birds. Here, we tested this using Raman spectroscopy to identify pigments in feathers exhibiting colors suspected of being produced by pheomelanin (i.e. dull red, yellow, greyish-brown and greenish-brown) in 26 species from the three main lineages of Psittaciformes. We detected the non-sulphurated melanin form (eumelanin) in black, grey and brown plumage patches, and psittacofulvins in red, yellow and green patches, but there was no evidence of pheomelanin. As natural melanins are assumed to be composed of eumelanin and pheomelanin in varying ratios, our results represent the first report of impairment of mixed melanin-based pigmentation in animals. Given that psittacofulvins also avoid the uptake of circulating carotenoid pigments, these birds seem to have evolved a capacity to avoid functional redundancy between pigments, likely by regulating follicular gene expression. Our study provides the first vibrational characterization of different psittacofulvin-based colors and thus helps to determine the relative polyyene chain length in these pigments, which is related to their antirestant protection activity.

**KEY WORDS:** Pheomelanin, Color redundancy, Plumage coloration, Polynes, Psittacofulvin, Raman spectroscopy

**INTRODUCTION**

Virtually all organisms have evolved pigmentation based on melanins, mainly owing to the benefits derived from their broadband absorbance properties and capacity to protect cells against the damaging effects of solar ultraviolet (UV) radiation (Brenner and Hearing, 2008). Animal melanins occur in two primary forms: eumelanin, polymers of indole units, and pheomelanin, oligomers of sulfur-containing heterocycles, containing sulfhydryl groups from the amino acid cysteine (Ito and Wakamatsu, 2008). This chemical heterogeneity is responsible for the optical properties of melanins, which provide animals with a wide diversity of colors ranging from black, brown and grey hues generated by eumelanin to reddish, orange and yellowish hues generated by pheomelanin (Galván and Wakamatsu, 2016). The biosynthesis of melanins is considered a mixed process that leads to the formation of both eumelanin and pheomelanin in varying ratios (Ito and Wakamatsu, 2008). Indeed, despite the existence of pheomelanin synthesis in fishes being unclear (Ito and Wakamatsu, 2003; Kotterl et al., 2015), eumelanin and pheomelanin are known to co-occur at different ratios in the integument of molluscs (Speiser et al., 2014), insects (Galván et al., 2015) and all vertebrates including humans (Ito, 2003; d’Ischia et al., 2015; Del Bino et al., 2015).

The apparent wide distribution of both melanin forms in animals suggests that mixed melanogenesis had an early evolutionary origin. This has probably been favored by the kinetics of the synthesis process, which consists of a ‘default’ pathway (i.e. in the absence of sulfhydryls) that leads to the production of eumelanin from the oxidation of the amino acid tyrosine and subsequent polymerization of intermediate compounds. However, sulfhydryl groups are always incorporated into this pathway, leading to the formation of pheomelanin, as long as cysteine is present in the cells (melanocytes in vertebrates) above a certain threshold concentration (Ito and Wakamatsu, 2008). Cysteine and its metabolites play a role in antioxidant protection; thus, cysteine is present in the cells (melanocytes in vertebrates) above a certain threshold concentration (Ito and Wakamatsu, 2008). Cysteine and its metabolites play a role in several essential processes, ranging from energy supplementation to antioxidant protection; thus, cysteine is prevalent in cells (Wu et al., 2004; Lambert et al., 2015; Bender and Martinou, 2016). Therefore, the kinetics of melanin synthesis seems to easily favor mixed melanogenesis in cells, and it is not likely that pheomelanin has experienced many evolutionary losses, if any. In fact, the presence of a unique form of melanin has not been reported in the pigmentation of any vertebrate class. Mixed melanogenesis seems to be prevalent in animals, most notably in vertebrates. However, nothing is known about possible evolutionary losses of the mixed pigmentation process within animal classes.

The plumage coloration of birds (Class Aves) is one of the most diverse phenotypes in nature, and melanins are the most abundant pigments that contribute to it (Galván and Solano, 2016). However, some orders or families of birds have evolved a biochemical ability to synthesize unique pigments, such as the porphyrins turacin and tuncoverdin in turacos (Order Musophagiformes) (Church, 1892), spheniscins in penguins (Order Sphenisciformes) (Thomas et al., 2013), vitamin A in tropical starlings (Family Sturniidae) (Galván et al., 2019) and psittacofulvins in parrots and allies (Order Psittaciformes) (Stradi et al., 2001). This exclusivity of pigments allowed the evolution of conspicuous color phenotypes in these birds, which in some groups such as parrots is associated with a strikingly high color diversity (Martin, 2002; Berg and Bennett, 2010). Some of the colors resulting from these exclusive pigments recall those resulting from melanins (Toral et al., 2008), and as pigment synthesis entails the use of limiting resources and physiological costs (Galván and Solano, 2015), here we hypothesize that the evolution of novel metabolic pathways to pigmentaion may have favored the loss of mixed melanin-based pigmentation owing to the benefits of reducing metabolic costs and the absence of benefits of the functional redundancy of pigments.
In particular, we predict that the exclusive evolution in psittaciform birds of polynye pigments called psittacofulvins, which create yellow, orange and red plumage coloration (McGraw and Nogare, 2005), may have promoted the evolutionary loss of pheomelanin, given that this pigment also creates (albeit duller) yellow, orange and reddish plumage coloration (McGraw and Nogare, 2005), may have promoted the evolutionary loss of plumage coloration based on other polyenic but dietary acquired pigments also able to create similar plumage coloration (i.e. carotenoids) despite being present at high circulating levels in parrots (McGraw and Nogare, 2004). An absence of pheomelanin in the feathers of parrots would represent the first report of impaired mixed melanogenesis in animals. Here, we tested this hypothesis using Raman spectroscopy to identify the pigments responible for plumage coloration in 26 species belonging to the three main lineages of Psittaciformes (Psittacoidea, Cacatuoidea and Strigopoidea) and displaying color hues suspected of being produced by pheomelanin, i.e. dull yellow, orange, reddish and brown coloration (Galván and Wakamatsu, 2016).

**MATERIALS AND METHODS**

**Species selection and feather sampling**

Psittaciform species were selected on the basis of plumage patches of colors suspected of being produced by pheomelanin. Although pheomelanin produces yellow, orange and red hues similar to those produced by pheomelanins, the colors produced by pheomelanin are not bright, but dull (Galván and Wakamatsu, 2016). Thus, we selected 26 psittaciform species whose plumage included a patch displaying dull yellow, orange, reddish or brown coloration, whose low level of brightness makes them likely candidates to be produced by pheomelanin (Fig. 1, Table 1). The selected species covered a high phylogenetic diversity, thus being a significant representation of the Order Psittaciformes (Fig. 2). For simplicity, the plumage patch colors are here described as red, yellow, green, grey and brown (Table 1).

The selection of species was made by examining book illustrations (Juniper and Parr, 2001) and with the advice of parrot captive breeders in Belgium and The Netherlands, who also provided feather samples. We included two mutations that appear in captivity in two species, the whiteface mutation of the cockatiel *Nymphicus hollandicus* and the opaline mutation of Bourke’s parrot, *Neopsephatus bourkii*, as mutated birds exhibit changes in plumage pigmentation potentially owing to the synthesis of pheomelanin (Martin, 2002) (Fig. 1). The whiteface mutation is inherited as an autosomal recessive mutation, while the opaline mutation is inherited as a sex-linked recessive mutation (Van den Abeele, 2016; van der Zwan et al., 2019).

One or two feathers from the studied plumage patches were collected from an adult bird of each species and analyzed for...
The main pigment identified in the studied color patches by means of Raman spectroscopy is indicated. When not specified, the indicated pigment was detected in both barbs and barbules of feathers. In the Cuban amazon (Amazona leucocephala), the chestnut-fronted macaw (Ara severus) and the black-cheeked lovebird (Agapornis nigrigenis), the color plumage patches are generated by a spatial segregation of psittacofulvin and eumelanin between barbs and barbules in feathers. The use of one or two samples from adults per species has been proven sufficient to characterize the pigmentation phenotype of bird species (Galván et al., 2018a). All feather samples were collected from birds bred and kept in captivity. The breeders had all necessary permits to hold the birds following guidelines from Belgian and Dutch authorities.

**Raman spectroscopy**

We used a Thermo Fisher DXR confocal dispersive Raman microscope (Thermo Fisher Scientific, Madison, WI, USA) operating at the National Museum of Natural Sciences (MNCN, CSIC) in Madrid, Spain, to identify the pigments responsible for the color of the studied feathers. Dispersive Raman spectroscopy can detect pigment molecules in solid samples at concentrations as low as 0.05–0.1% (w/w) (Bouffard et al., 1994; Massonnet et al., 2012). The system has a point-and-shoot Raman capability of 1 µm spatial resolution. We used an excitation laser source at 780 nm and a slit aperture of 25 µm to analyze the wavenumber range of 300–2500 cm\(^{-1}\). When the samples contained psittacofulvin, we used a 50× confocal objective lens, a laser power of 7 mW, and an integration time of 3 s and 12 accumulations. When the samples contained melanins, to avoid the burning of samples owing to their darker color and to optimize results, we used a 100× confocal objective lens, a laser power of 1 mW, and an integration time of 3 s and 30 accumulations. The system was operated with Thermo Fisher OMNIC 8.1 software. We analyzed one to two feathers from one bird of each species. One barb and one barbule chosen at random were analyzed in each feather. The average Raman spectra corresponding to each pigment were calculated for each species. The computational analysis including importing and pre-processing (baseline correction and normalization) of data was performed with MATLAB® R2014b (MathWorks Inc., Natick, MA, USA) using the PLS Tollbox version 7.9.3 (Eigenvector Research Inc., USA). The identification of pigments was made on the basis of diagnostic vibrational bands. The Raman spectrum of eumelanin shows defined Raman bands at 1380 and 1580 cm\(^{-1}\) resembling the D and G bands characteristic of disordered graphite, in addition to a weaker band at 500 cm\(^{-1}\) (Huang et al., 2004; Galván et al., 2013a, 2018b). In contrast, the Raman spectrum of pheomelanin shows wide Raman bands at approximately 500, 1490 and 2000 cm\(^{-1}\) owing to the vibration of the methyl group; however, this is present in the Raman spectra of other polyenic pigments such as carotenoids (Maia et al., 2014; Fernandes et al., 2015).

**RESULTS**

We found Raman signals of eumelanin in nine of the species or mutations included in the study (Fig. 3). These corresponded to three species with grey plumage patches and two species with brown plumage patches (Table 1, Fig. 1). Additionally, eumelanin was found spatially segregated in the red or brown plumage patches of other three species (the Cuban amazon, Amazona leucocephala, the chestnut-fronted macaw, Ara severus, and...
the black-cheeked lovebird, Agapornis nigrigenis) (Fig. 1), in which eumelanin was found in black or grey barbules while psittacofulvin was found in red barbs (Table 1). In contrast, no Raman signal of pheomelanin was found in any of the plumage patches analyzed. This indicates that pheomelanin is not present in the feathers of psittaciforms, or that it is present in non-significant amounts.

Raman signals of psittacofulvin were detected in all red plumage patches analyzed, corresponding to 18 species (Fig. 4, Table 1). Psittacofulvins were also found to cause dull yellow plumage coloration in two species (Fig. 5A) and green coloration in six species (Fig. 5B). Psittacofulvin was also found to be the pigment causing dark brown coloration in two species (Fig. 5D), the brown lory (Chalcopsitta duivenbodei) and the black-cheeked lovebird (Fig. 1), though in the latter species the brown plumage patch is produced by a combination of red psittacofulvin-containing barbs and grey eumelanin-containing barbules (Table 1). However, the Raman spectra of yellow, green and brown plumage patches were remarkably similar, all showing the two diagnostic bands of psittacofulvin. Variation between color groups in the frequency of the first band (∼1130 cm$^{-1}$) was notably low, as it was located at 1131 cm$^{-1}$ in the spectra of red feathers, 1134 cm$^{-1}$ in the spectra of yellow feathers, 1136 cm$^{-1}$ in the spectra of green feathers and 1133 cm$^{-1}$ in the spectra of brown feathers (Fig. 6). However, variation was more marked in the frequency of the second band (∼1530 cm$^{-1}$), being located at 1525 cm$^{-1}$ in the spectra of red feathers, 1531 cm$^{-1}$ in the spectra of yellow feathers, 1536 cm$^{-1}$ in the spectra of green feathers and 1528 cm$^{-1}$ in the spectra of brown feathers (Fig. 6).

**DISCUSSION**

Our results show that pheomelanin does not contribute to the plumage pigmentation of psittaciform birds, despite assumptions made in some previous studies lacking analytical evidence (Tinbergen et al., 2013; Delhey and Peters, 2017). As we included in our analyses a significant representation of psittaciform species with plumage colors suspected of being produced by pheomelanin (Galván and Wakamatsu, 2016) and from a high phylogenetic diversity that included the three main lineages of Psittaciformes, these results provide evidence that psittaciform birds synthesize eumelanin to pigment feathers black, grey and brown, but do not synthesize pheomelanin or synthesize it at negligible amounts. As natural melamins are considered mixed pigments in which eumelanin and pheomelanin are present in varying ratios (Ito and Wakamatsu, 2008),
parrots represent the first animals in which an impaired mixed melanin-based pigmentation system is reported. The presence of pheomelanin (and eumelanin) in organisms such as molluscs and insects (Speiser et al., 2014; Galván et al., 2015) and in all vertebrates (d’Ischia et al., 2015; Kottler et al., 2015) suggests that the absence of pheomelanin in psittaciform birds is an exclusive evolutionary loss in this order within Aves. Indeed, no other birds are known to lack pheomelanin in their feathers (Galván and Solano, 2016).

Psittaciform birds have evolved an exclusive pigmentation system by expressing the MuPKS gene, which codes for a
polyketide synthase (Cooke et al., 2017), in feather follicles. This leads these birds to synthesize polyene pigments called psittacofulvins that produce colors that may be similar in hue to those produced by pheomelanin (Toral et al., 2008; Galván and Wakamatsu, 2016). Given this functional redundancy and that pheomelanin synthesis may be physiologically limiting under environmental stress because it reduces GSH availability in melanocytes (Galván, 2018), and that psittacofulvin synthesis does not seem to imply any comparable physiological limitation, we hypothesized an impairment of mixed melanin-based pigmentation in psittaciforms. Indeed, psittaciforms do not deposit carotenoids (dietary polyene pigments very commonly causing plumage coloration in other birds) in feather follicles, despite circulating them through the blood at high levels (McGraw and Nogare, 2004). The psittacofulvin-based pigmentation system of psittaciforms thus seems to have blocked the physiological activity of other pigmentation systems.

Interestingly, our results show that psittaciforms do not synthesize pheomelanin, but synthesize eumelanin, which produces dark colors (black, grey and dark brown) not resembling those produced by psittacofulvins (Toral et al., 2008; Galván and Wakamatsu, 2016). This makes it likely that the avoidance of functional redundancy between pigments is the evolutionary cause that has favored the impairment of the mixed melanin-based pigmentation system in psittaciforms. This impairment might be exerted through a regulation of the expression of genes controlling pheomelanin synthesis in melanocytes at the dermal papillae of feather follicles (Lin et al., 2013). Candidate genes in this regulation are those coding for the antagonist peptides of the melanocortin 1 receptor in the membrane of melanocytes, namely agouti-signalling (ASIP) and agouti-related (AGRP) proteins (Nadeau et al., 2008), and also genes that control the availability of cysteine in melanocytes such as that encoding the cystine/glutamate antiporter xCT (Slc7a11; Chintala et al., 2005) and that encoding
cystinosin (CTNS; Town et al., 1998). The activation of the MuPKS gene in feather follicles, which is observed in psittaciforms but not in other birds (Cooke et al., 2017), has thus probably led to a co-regulation of other genes, impairing the synthesis of pheomelanin and the uptake of circulating carotenoids (McGraw and Nogare, 2004), and promoting a selectivity for the psittacofulvin-based pigmentation system. Interestingly, this likely gene regulation mechanism in feather follicles would parallel the loss of gene duplicates that is observed when they exhibit functional redundancy (Lynch and Conery, 2000; Cooke et al., 2017). Future studies should investigate this gene regulation mechanism leading to a prevailing pigmentation system.

Our study provides the first vibrational characterization of psittacofulvins giving rise to different plumage colors. Psittacofulvins giving rise to yellow plumage are composed of polyene chains of 14, 16 and 18 carbon atoms in the form of conjugated fatty acids, whereas psittacofulvins giving rise to red plumage are probably synthesized by reducing yellow ones to form fully conjugated aldehydes of 14, 16, 18 and 20 carbon atom chains (Stradi et al., 2001; Cooke et al., 2017). Psittacofulvins giving rise to green plumage are composed of yellow psittacofulvins, combined with a structural effect of feather morphology (Cooke et al., 2017). Our results show that psittacofulvins can also give rise to brown plumage coloration when combined with eumelanism. We found great similarity in the vibrational spectra of psittacofulvins of these color groups, which suggests that these colors are produced by similar mixtures of polyenes of different chain length, as already shown for yellow and red psittacofulvins (see above), but that certain chain length values must prevail in each color group to give rise to the observed differences in the absorbance properties, i.e. colors. This is supported by a close examination of the frequency of the two Raman bands of psittacofulvins. Although the band at approximately 1130 cm⁻¹, which is due to C=C stretching in the polyene chain, is remarkably constant between species and plumage color groups, the band at approximately 1530 cm⁻¹, which is due to C=C stretching (Maia et al., 2014), is more variable between groups (see Fig. 6). C=C stretching depends on the length of the polyene chain (Maia et al., 2014), and accordingly, a clear differentiation can be observed in the frequency of C=C stretching of red psittacofulvins and that of yellow and green psittacofulvins (Fig. 6), which lack C₂₀ chains (Cooke et al., 2017). This observation is physiologically relevant, because psittacofulvins are good electron acceptors (Martínez, 2009), and this antirreductant activity seems to increase with chain length in at least polyenals (Tagliazucchi et al., 2006). This suggests that psittacofulvins giving rise to red plumage may exert a lower cellular protection activity than those giving rise to yellow or green plumage, or that psittacofulvins giving rise to a given color (e.g. yellow) but composed of polyenes of different chain lengths may differ in cellular protection capacity. Our results show that Raman spectroscopy may represent a useful non-invasive tool to determine the relative chain length of psittacofulvins and thus investigate their physiological potential as antirreductant compounds.

In conclusion, psittaciform birds have evolved an impairment of the mixed melanin-based pigmentation system by blocking, probably by a regulation of genes involved in melanogenesis in follicular melanocytes, the synthesis of pheomelanin. We propose that this impairment, which adds to the blocking of the uptake of circulating carotenoids by feather follicles in these birds, has likely been favored by a functional redundancy between pigments. Given the apparent solution to this redundancy shown here, it could be possible that birds may modulate the activity of feather follicles to maximize the diversity of color phenotypes while minimizing the use of pigment resources. Future studies should provide further details about the physiology of psittacofulvin synthesis to determine whether a comparison of physiological implications between pigments can explain the exclusive prevalence of the psittacofulvin-based pigmentation system in Psittaciformes.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.C.d.O.N., I.G.; Formal analysis: A.C.d.O.N., I.G.; Investigation: A.C.d.O.N., I.G.; Resources: D.V.d.A.; Writing - original draft: A.C.d.O.N., I.G.; Visualization: A.C.d.O.N.; Supervision: I.G.; Funding acquisition: D.V.d.A.

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