



Multiple ways to become red: Pigment identification in red feathers using spectrometry

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ABSTRACT

Red hues are a challenge in studies on the evolution of bird coloration, as multiple pigments such as carotenoids, pheomelanin, psittacofulvins, porphyrins, turacin, haemoglobin and even exogenous iron-oxides, may confer red colors. Determining the pigment type is paramount and here we investigate the differences in spectrum reflectance for six pigments resulting in red colorations in feathers of different species, with a focus on discriminating among melanins and carotenoids. Pigment chemical identification was obtained from the literature or using High Performance Liquid Chromatography (HPLC) in our laboratory. We have also derived discriminant formulas for identification of the major known types of pigments based on parameters of the reflectance curves obtained with a portable spectrometer. Our results indicate that the reflectance patterns of coloration perceived as red patches widely differ. The distinction between carotenoid- and melanin-based reflectance curves is relatively straightforward: sigmoid versus straight slope. The spectral reflectance curves of feathers containing red psittacofulvins are sigmoid, whereas iron oxide and porphyrin-containing feathers recall pheomelanins in rendering a straight slope. In the case of turacin-based coloration, the spectral shape is unique. For the pigments with enough number of species sampled (i.e., carotenoids, melanins and psittacofulvins) the differences in reflectance shape are important enough to allow separation of carotenoid and melanin derived colorations based on reflectance curves alone.

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1. Introduction

Feather patterns and coloration can be adaptive in a number of ways (see, e.g., Hill and McGraw 2006, and references therein), as they may be responsible for camouflage, play a role in intra- or inter-specific communication, or serve physiological functions, such as thermoregulation (Margalida et al., 2008), solar UV-rays defence, resistance to abrasion or bacterial damage (Grande et al., 2004). All those functions are achieved through either feather structure, as with white, blue and iridescent colors (Prum, 2006), and/or through the use of a rather limited array of pigments. In this respect, the majority of bird species rely on melanins, of which there are only two basic types: pheomelanin and eumelanin. The carotenoids follow in prevalence in the avian integument. These pigments have been described in the feathers or skin of about 150 species in seven orders, but are presumed to be in many others (McGraw, 2006a). Pigments other than melanins and carotenoids are rarely found in feathers, and include, for instance, porphyrins in the plumage of owls and bustards, and psittacofulvins in parrots (McGraw, 2006b).

Eumelanin renders black or grey feather coloration. Pheomelanin, in turn, is responsible for brown, yellowish, chestnut and red colors. If black color in a feather is synonymous of presence of melanin, as no other black pigment has been described so far (McGraw, 2006c), red color is a challenge for researchers, as carotenoids, pheomelanin, psittacofulvins, porphyrins, turacin and even exogenous iron-oxides (Negro et al., 1999; Negro and Margalida, 2000; Montgomerie, 2006a) may color the feathers red. For instance, early researchers attributed the cosmetic reddish tinge of the ventral areas of bearded vultures to carotenoids (Negro et al., 1999). Still others have reported that the dark red color in the throat patch of Barn Swallows (*Hirundo rustica*) was due to carotenoid pigments (Stradi, 1998; Saino et al., 1999), only to discover later on that it was due to melanin (McGraw et al., 2004a, b). Determining the pigment type in behavioural studies is paramount, as, e.g., melanins and carotenoids have different properties and associated production costs (McGraw, 2006a). Melanin is synthesized from tyrosine and, except for the rare albinos (McGraw, 2006c), all birds seem to be capable to produce it. At present, most evidence indicates that melanization is not dependent on the diet (Hill and McGraw, 2003). Carotenoids, on the other hand, need to be derived from the food, as these pigments cannot be synthesized by vertebrates, but are necessary for the synthesis of vitamins, to boost the immune system and to control oxidative stress, and ample evidence indicate that carotenoid-based coloration is condition-dependent (Hill, 2006).

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Here we investigate the differences in spectrum reflectance for six pigment types resulting in red colorations of different species. Red colors, here encompassing different hues in the longer wavelengths of the visible spectrum and thus including ferruginous tinges, as well as pink, orange, crimson and vermillion, are widely distributed in the plumage (Hill, 1996) and exposed skin (Negro et al., 2005) of birds belonging to species in distantly related orders. Even though we are not aware of any study on the frequency of red colors in different habitats or in birds with different ecologies (but see Gray, 1996), red hues do not seem to be linked to any particular latitude, habitat, diet or bird size. As such, red colors can be found in small passerines such as the vermillion flycatcher (*Pyrocephalus rubinus*), an inhabitant of open areas; in medium-sized forest birds such as the red and green macaw (*Ara chloroptera*) or in very large wetland birds, such as the flamingos (*Phoenicopterus spp.*).

Given the importance of assigning feather pigments to the major pigmentary groups, our aim has been to present spectral reflectance curves of feathers containing different but chemically well characterised red pigments. We have also derived discriminant formulas for identification of the major known types of pigments (i.e., pheomelanins, carotenoids and psittacofulvins) based on parameters of the reflectance curves obtained with a commercial, easy-to-use, portable spectrometer. The distinction between carotenoid- and melanin-based reflectance curves has already been established: carotenoids render a sigmoid pattern whereas melanins render a straight slope (e.g., Andersson et al., 2007). However, reflectance spectra of all red pigments in the plumage of birds have not been analysed concurrently. As chemical (and thus uncontested) pigment identification in the literature covers relatively few species, we analysed feather pigment content by HPLC in our laboratory for a range of species lacking previous pigment identification.

2. Material and methods

We measured spectral reflectance in red color patches of 54 species from 30 families and 13 orders (Table 1). To judge a color as “red” we based our choice in our own experience but also on descriptions by other authors of bird identification guides (see Table 1). The species were chosen among the ones available as study skins in the Vertebrate Collection of the Doñana Biological Station (CSIC). This collection is mainly composed of species from the Western Palearctic and South America. The sampled skins were prepared between 1954 and 2002, except one specimen from 1939. Older museum skins may suffer color changes (see, e.g., Mullen and Pohland, 2008 and references therein), but mainly in the UV range and only locally due to the action of fluorescent preservation agents (Pohland and Mullen, 2006). The specimens we handled had not suffered from any obvious change in feather coloration and there is no reason to believe the pigments had been altered significantly.

2.1. Recording spectral reflectance

Color was recorded from 4 June to 23 July 2004 on the red patches of the plumage using a Minolta CM-2600d portable spectrometer (Minolta Co., Ltd., Osaka, Japan) with UV (xenon flashlight source) and visible light (standard illuminant D65). Reflectance curves were obtained by the emission of a light flash of known characteristics, and the measurement of maximum reflectance at 10 nm intervals between 360 and 740 nm, which is the visible range plus or minus 40 nm. We obtained spectrometer measurements from up to three specimens for each bird species (two individuals for two species and one for seven species). In the case of species with strong plumage sexual dimorphism resulting in differences in feather pigmentation among genders, measurements were taken from up to three males and three females (i.e., *Eudocimus ruber*). In addition, and in order to obtain accurate values of reflectance, we repeated spectrometer

measurements three times on the same area of the plumage surface. The spectrometer measurement was taken from the most saturated zone of the red color patch of each specimen, considering a color patch as an area of continuous visual coloration greater than 3 mm², which is the area covered by the probe of our spectrometer. When differences in plumage brightness among specimens from the same species were evident, we selected those three individuals with the freshest (less damaged) looking plumage.

2.2. Extraction of feather pigments for chemical identification

Pigment type was assigned according to the literature if the species had already been analysed chemically (see Table 1). For the remaining species, we determined feather type in our own laboratory. These feathers with unknown pigments were first subjected to an extraction procedure for carotenoids, as follows: 0.005–0.0012 g of feather sample (ca. 12 feathers) were placed within a folded filter paper and introduced into a 10 mL test tube for subsequent extraction. Ten mL of *N, N*-dimethylformamide were added and the tube was placed at 60 °C for 60 min, including sonication for 5 min every 30 min. All fractions were pooled in a decanting funnel and treated with 50 mL of diethyl ether to collect the pigments. 10% NaCl was added to allow the separation of the phases. The ether phase was filtrated through anhydrous sodium sulphate, evaporated in a rotary evaporator until sample dryness, and taken up to 0.2 mL of acetone. Resulting extracts were stored at –20 °C until analysed by high-performance liquid chromatography (HPLC).

If the feathers actually contained carotenoids, the organic extraction described above changed feather color to whitish if no other pigment was present, or blackish if melanin was also co-occurring in the feather. Otherwise, as in pheomelanin-containing feathers, the organic extraction did not change feather color significantly.

2.3. Carotenoid identification

HPLC was carried out at the Chemical Ecology Laboratory of Doñana Biological Station using a Jasco PU-2089 Plus instrument equipped with a quaternary pump (Jasco Analítica Spain, S.L.). Carotenoid analyses were carried out by using a reverse phase C₁₈ column (Phenomenex Synergi 4 μ) and a precolumn of the same material with a particle size of 5 μm. Samples were pre-filtered using OEM filter Nylon (0.45 μm 4 mm) and later injected with a Rheodyne 7725i Valve equipped with a 20 μL loop (Rheodyne, Rohnent Park, CA, USA). The eluent system was as the one described in Mínguez-Mosquera and Hornero-Méndez (1993), except that the flow rate was 1 mL min^{–1}. Data were acquired between 195 nm and 650 nm with a multiwavelength detector MD-2010 Plus (Jasco Analítica Spain, S.L.).

Quantification of carotenoids, even though it was not crucial for this study, was performed using reference astaxanthin. Known dilutions of astaxanthin were injected in the HPLC instrument to build a calibration curve at 450 nm. Concentration of individual carotenoids was calculated from HPLC areas recorded at 450 nm.

2.4. Identification of melanins, psittacofulvins and porphyrins

To determine the presence of melanins we extracted the feathers with NaOH (McGraw, 2006c), and centrifuged the solution to get soluble pheomelanin (brownish in appearance) in the supernatant and a black pellet containing eumelanin. All species contained both pheomelanin and eumelanin in variable proportions in the red feathers, as previously reported (McGraw, 2006c). The melanin protocol applied to parrot feathers containing psittacofulvins (McGraw and Nogare, 2004 and 2005) rendered a yellow supernatant with a distinct absorption spectrum in the range 400–500 nm, very different to the one of phaeomelanins (see, e.g., McGraw, 2006c). The previously unknown porphyrin of *Elanus caeruleus* (see Table 1) was

Table 1

Pigment identification in specific feather tracts of the bird species considered in this study

Order	Family	Species	Body part and color	Pigment	Reference
Podicipediformes	Podicipedidae	<i>Podiceps cristatus</i>	Chestnut foreneck	Melanin	This study
Ciconiiformes	Ardeidae	<i>Bubulcus ibis</i>	Orange nuptial plumage in breast	Melanin	This study
	Threskiornithidae	<i>Eudocimus ruber</i>	Red covert feather	Carotenoid	Trams (1969)
Phoenicopteriformes	Phoenicopteridae	<i>Phoenicopiterus ruber</i>	Red covert feather	Carotenoid	Fox and Hopkins (1966)
Anseriformes	Anatidae	<i>Anas clypeata</i>	Chestnut flanks	Melanin	This study
Falconiformes	Accipitridae	<i>Elanus caeruleus</i>	Rufous breast in juveniles	Porphyrin	This study
		<i>Gypaetus barbatus</i>	Rufous buff underbody	Iron oxide	Negro et al. (1999)
	Falconidae	<i>Falco subbuteo</i>	Red 'trousers' on legs	Melanin	This study
Gruiformes	Turnicidae	<i>Turnix sylvatica</i>	Orange breast patch	Melanin	This study
Charadriiformes	Charadriidae	<i>Vanellus vanellus</i>	Orange undertail coverts	Melanin	Bókonyi et al. (2003)
	Scolopacidae	<i>Calidris canutus</i>	Rufous orange breast	Melanin	This study
		<i>Limosa limosa</i>	Rufous orange breast	Melanin	This study
Pterocliiformes	Pteroclididae	<i>Pterocles alchata</i>	Chestnut band on breast	Melanin	This study
Psittaciformes	Psittacidae	<i>Eos reticulata</i>	Red rump	Psittacofulvin	McGraw and Nogare (2005)
		<i>Lorius garrulus</i>	Red breast	Psittacofulvin	This study
		<i>Platycercus eximius</i>	Red breast	Psittacofulvin	McGraw and Nogare (2005)
		<i>Psittacus erithacus</i>	Bright red tail	Psittacofulvin	McGraw and Nogare (2005)
		<i>Ara macao</i>	Red wing	Psittacofulvin	McGraw and Nogare (2005)
		<i>Ara chloroptera</i>	Red rump	Psittacofulvin	McGraw and Nogare (2005)
		<i>Ara rubrogenys</i>	Orange-red shoulder patch	Psittacofulvin	This study
		<i>Ara severa</i>	Red shoulder	Psittacofulvin	McGraw and Nogare (2005)
		<i>Aratinga acuticaudata</i>	Reddish inner webs of tail	Psittacofulvin	McGraw and Nogare (2005). This study
		<i>Pionus menstruus</i>	Red undertail coverts	Psittacofulvin	McGraw and Nogare (2005). This study
Cuculiformes	Musophagidae	<i>Tauraco macrorhynchus</i>	Crimson primaries	Turacin	Rimington C. (1939)
	Cuculidae	<i>Clamator glandarius</i>	Bright rusty brown primaries	Melanin	This study
Coraciiformes	Alcedinidae	<i>Alcedo atthis</i>	Orange brownish-red underparts	Melanin	This study
	Coraciidae	<i>Coracias garrulus</i>	Reddish brown back	Melanin	This study
	Upupidae	<i>Upupa epops</i>	Buff-pink crest	Melanin	This study
Piciformes	Picidae	<i>Dendrocopos major</i>	Deep red undertail coverts	Carotenoid	Stradi et al. (1998)
		<i>Picus viridis</i>	Red crown	Carotenoid	Stradi et al. (1998)
Passeriformes	Cotingidae	<i>Rupicola peruviana</i>	Bright orange mantle	Carotenoid	This study
	Hirundinidae	<i>Hirundo rustica</i>	Blood-red throat	Melanin	McGraw et al. (2004a)
	Turdidae	<i>Turdus iliacus</i>	Rusty red underwing	Melanin	This study
	Sylviidae	<i>Sylvia cantillans</i>	Brick-red throat	Melanin	This study
	Muscicapidae	<i>Erithacus rubecula</i>	Rusty red 'bib'	Melanin	Tella et al. (2004)
		<i>Luscinia megarhynchos</i>	Rusty red tail	Melanin	This study
		<i>Luscinia svecica</i>	Rusty red tail base	Melanin	Tella et al. (2004)
		<i>Phoenicurus phoenicurus</i>	Orange-red rectrices	Melanin	This study
		<i>Saxicola torquata</i>	Orange breast patch	Melanin	Tella et al. (2004)
	Tichodromidae	<i>Tichodroma muraria</i>	Blood-red scapulars	Carotenoid	Stradi et al. (1998)
	Laniidae	<i>Lanius senator</i>	Red-brown crown	Melanin	This study
	Ploceidae	<i>Malimbus nitens</i>	Red breast	Carotenoid	This study
		<i>Euplectes hordeaceus</i>	Red rump	Carotenoid	This study
	Estrildidae	<i>Pyrenestes ostrinus</i>	Brilliant crimson-red breast	Carotenoid	This study
		<i>Spermophaga haematina</i>	Bright red breast	Carotenoid	This study
	Fringillidae	<i>Loxia curvirostra</i>	Red breast	Carotenoid	Stradi et al. (1996)
		<i>Carduelis cucullata</i>	Bright orange-vermilion breast	Carotenoid	This study
		<i>Carduelis cannabina</i>	Red breast	Carotenoid	Stradi et al. (1997)
		<i>Carduelis carduelis</i>	Red face	Carotenoid	Stradi et al. (1995)
		<i>Pyrrhula pyrrhula</i>	Bright red breast	Carotenoid	Stradi et al. (2001)
	Thraupidae	<i>Tangara arthus</i>	Red breast	Carotenoid	This study
	Icteridae	<i>Sturnella supercilialis</i>	Bright red breast	Carotenoid	This study
		<i>Icterus icterus</i>	Orange rump	Carotenoid	This study
		<i>Amblyramphus holosericeus</i>	Brilliant orange-red breast	Carotenoid	This study

"Reference" includes the source for chemical identification: either our own analysis (this study) or data reported by previous authors. Color descriptions for European birds as given in the Collins Bird Guide (Svensson et al., 2000). The remaining descriptions as given in the Handbook of the Birds of the World (Del Hoyo et al., 2004); The Birds of Africa (Fry and Keith, 2004) and The Birds of South America (Ridgely and Tudor, 1994).

analysed by both HPLC and Thin-Layer Chromatography (TLC), and identified with appropriate controls.

2.5. Statistical analyses

A principal component analysis (PCA) based on reflectances at 10 nm intervals in the 360–740 wavelength segment contained in the spectral reflectance curves was carried out to characterise reflectance patterns of the different pigments. Measurements of all specimens ($n=140$) were included in the analysis, with no prior calculations of means for each species. PCA is the most commonly used multivariate method to analyse reflectance spectra in bird color research (Montgomerie, 2006b) and summarises most of the variability in reflectance spectra in a reduced number of variables derived as lineal

combinations of the different variables measured. A GLM including the variable "species" as a random factor was performed to compare the scores of the four first components of the PCA to determine whether there were significant differences between pigments. Ranked values of the variables "Factor 1" and "Factor 2" were used to attain normality (see Conover and Iman, 1981).

Subsequently, a discriminant analysis was done by stepwise forward selection of reflectance at the 360–740 wavelengths intervals. For this analysis, mean readings for every species at each wavelength were used at 10 nm intervals. Pigment type was incorporated as a categorical variable. Variable selection was based on Wilks lambda and F value. With this approach we derived a discriminant formula based in reflectance in the minimum number of wavelengths intervals. The reliability of the derived formula was estimated by a

jackknife (i.e., leave-one-out) statistical procedure (Tabachnick and Fidell, 2001).

3. Results

3.1. Patterns of variation in reflectance spectra

Typical reflectance spectra for the six different red pigments considered in this study are given in Fig. 1. For carotenoids, melanins and psittacofulvins we randomly chose one spectrum among the available species. For the rare pigments, i.e. iron-oxides, porphyrin and turacin, only one species was available for study (see legend in Fig. 1). The distinction between carotenoid- and melanin-based reflectance curves is relatively straightforward: sigmoid versus straight slope. The spectral reflectance curves of feathers containing red psittacofulvins are sigmoid, whereas iron oxide- and porphyrin-containing feathers recall pheomelanins in rendering a straight slope. In the case of turacin-based coloration, the spectral shape is unique, with very low reflectance from 360 to 590 nm, and a non-straight slope up to 740 nm.

Given the importance of telling apart at least the major types of pigments (i.e., carotenoids, melanins and psittacofulvins), we have developed discriminant formulas based on reflectance recordings at particular wavelengths. Four components with eigenvalues larger than 1 were extracted from the PCA based on the 360–740 wavelength segment analysed. The first component summarized 54.7% of the variance; the second component summarized 33.2%; the third component 8.2% and the fourth, 2.6% (see Fig. 2).

A GLM comparing principal component scores identified highly significant differences between pigments for the second ($F_{2,87}=29.24$; $p=0.0001$) and fourth ($F_{2,87}=6.06$; $p=0.003$) components of the PCA. Melanins had a significant lower second component scores than carotenoids and psittacofulvins (melanins = -0.71; carotenoids = 0.64; psittacofulvins = 0.60) and carotenoids had a significant lower fourth component scores than psittacofulvins (carotenoids = -0.21; psittacofulvins = 0.45). No differences were found for the first component ($F_{2,87}=0.44$; $p=0.6$) and third component of the PCA ($F_{2,87}=2.11$; $p=0.1$).

3.2. Classification of coloration according to spectra

In a preliminary analysis, we attempted a joint analysis of carotenoid, melanin and psittacofulvin derived coloration, but 9.8% (5/51) of species were classified in the wrong group. Subsequently we

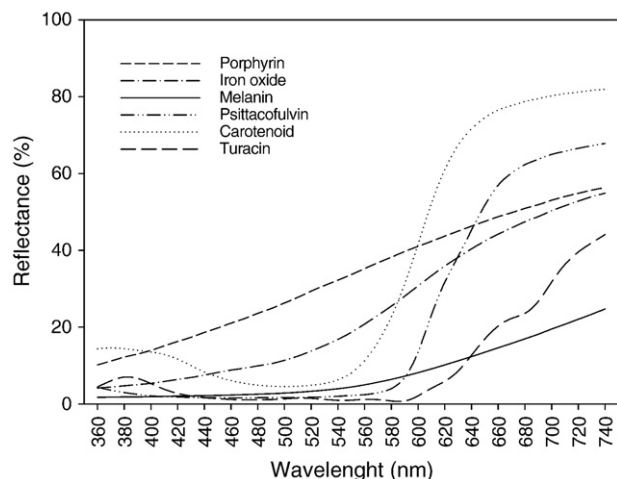


Fig. 1. Reflectance spectra for the different pigments producing red colorations considered in this study. Each spectrum corresponds to an actual reading on study skins of selected species as follows (the feather tracts involved are described in Table 1): Pheomelanin: *Hirundo rustica*. Carotenoid: *Phoenicopeterus ruber*. Psittacofulvin: *Ara macao*. Iron oxide: *Gypaetus barbatus*. Porphyrin: *Elanus caeruleus*. Turacin: *Turacus macrorhynchus*. Measurements were taken with a Minolta CM-2600d spectrometer.

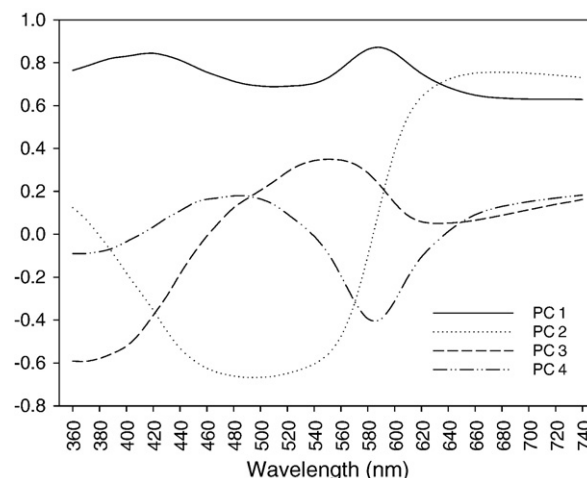


Fig. 2. Factor loadings for each variable on the principal components. Factor loadings reflect the relative contribution of each variable to the principal components scores. Values close to 1 or -1 indicate a stronger positive or negative relationship with the respective principal components analysis scores. As an example, for factor 1, reflectance at all wavelengths contributes positively to the scores. However, for factor 2, reflectance over 640 nm contributes positively whereas reflectance in the range 400–600 nm contributes negatively to factor 2 scores.

derived separate formulas using only two of the pigments in each analysis. These resulted in three different formulas.

The first formula separated with 0% error carotenoids from melanins based on reflectance measurements at four different wavelengths:

$$F1 \ D1 = -0.464 * \lambda_{520} - 2.092 * \lambda_{640} + 4.493 * \lambda_{660} - 2.214 * \lambda_{740} + 7.851$$

Where $D1 > 0$ for carotenoids and $D1 < 0$ for melanins.

The second formula was calculated for melanin and psittacofulvin derived colorations. 96.9% (31/32) of all the species were classified correctly as a function of their reflectance values at three wavelengths:

$$F2 \ D2 = 1.279 * \lambda_{560} - 4.314 * \lambda_{670} + 3.364 * \lambda_{720} + 7.788$$

Where $D2 > 0$ for melanins and $D2 < 0$ for psittacofulvins.

It was not possible to derive a formula to separate carotenoids from red psittacofulvins because there was no variable selected based on Wilks lambda and F value, suggesting that the reflectance spectra were too similar to allow reliable separation. Consequently, a coloration classified as melanin using the two previous formulas can be assumed to be derived from melanins. Additionally, and given that psittacofulvins are restricted to the order Psittaciformes, we can safely assume that out of this group there is no confusion, and that non-melanistic red colors in bird orders other than the Psittaciformes are thus carotenoids.

4. Discussion

Birds in numerous and phylogenetically unrelated species (see Table 1 and below) exhibit a wide array of red-colored integumentary patches, most often in sexually-mature individuals and/or only in one gender (typically the males). In general, those red patches are small relative to the size of the bird, and include, for instance, crimson head patches in woodpeckers (Family Picidae) or cranes (Genus *Grus*), the supraorbital erectile skin patches in grouse (Family Tetraonidae), epaulets in *Agelaius* species, the bill and tarsi in species such as the chough (*Pyrrhocorax pyrrhocorax*) or the white stork (*Ciconia ciconia*), or the tail of the African Grey Parrot *Psittacus erithacus*. These colorations can be generated by different biochemical pathways that are likely to affect the costs and trade-offs involved in the synthesis of these patches.

Our results indicate that the reflectance patterns of coloration perceived as red patches by humans widely differ. As already stated (e.g., McGraw, 2006c; Andersson et al., 2007), the distinction between carotenoid- and melanin-based reflectance curves is relatively straightforward (see Introduction). We expand their results by showing that the spectral reflectance curves of feathers containing red psittacofulvins are sigmoid, whereas iron oxide and porphyrin-containing feathers recall pheomelanins in rendering a straight slope. In the case of turacin-based coloration, the spectral shape is unique, with very low reflectance at low wavelengths and a non linear increase up to 740 nm. For the pigments with enough number of species sampled (i.e., carotenoids, melanins and psittacofulvins), the differences in reflectance shape are important enough to allow separation of carotenoid and melanin derived colorations based on reflectance curves only.

Interestingly, and even admitting that some evidence is contradictory (see, e.g., Westneat, 2006), birds seem to have a preference for red hues in contexts where visual communication is at stake, such as mate choice (Hill, 1991, 2006) and agonistic encounters (Pryke and Griffith, 2006; Negro et al., 1999). Piersma and Jukema (1993) proposed that red breasts in the bar tailed godwit (*Limosa lapponica*) were honest signals of migratory quality. However, the red signal is often confined to a reduced portion of the integument, strongly suggesting a production or, at least, maintenance cost. This idea had already been advanced by Hill (1996), but mainly for red carotenoids, that this author considered energetically more costly to produce than yellow or orange carotenoids. The fact that red plumage colors may be obtained from so varied pigment sources, can also be seen as additional proof of both preference and costliness of this particular hue. Some species resort to diet and some physiological adaptations to become red (i.e., those with carotenoid-dependent coloration, as in the Fringillidae), others synthesize the red pigments by themselves (as in species producing psittacofulvins or porphyrins); a few combine yellow carotenoids and brown pheomelanin, as in the epaulets of *Agelaius* species (McGraw, 2006a), some circulate haemoglobin – again an endogenously generated pigment – through the skin (as in the Ostrich, see Negro et al., 2005), and still others get the pigments directly from the environment by bathing in red soils (e.g., Bearded Vultures – Negro et al., 1999; Sandhill Cranes *Grus canadensis* – Montgomerie, 2006a).

How a preference for red hues may have evolved in so many avian species warrants further study due to its evolutionary implications (see e.g., Hill, 1996). Perhaps it is due to a sensory bias towards longer wavelengths, as recently reported for primates with trichromatic color systems (Fernández and Morris, 2007), because red, due to its high saturation and chroma, is very efficiently perceived against a background of green leaves or a blue sky, or because it is the ultimate hue in terms of costliness (thus ideally suited to be sexually selected). In any case, the first step for tackling the above question on red high prevalence is to sort out the different pigments involved in red color production. Here we have provided a tool for identifying the main red pigments in the plumage, based on their spectral reflectance. Although chemical methods remain as the “gold standard” for pigment identification, portable spectrometry is ideal for researchers working in the field since it does not require a laboratory and associated drawbacks. It does not generate any hazardous residues and, as we have shown, provides direct pigment identification without the need for feather collection, storage and transportation (often requiring special permits such as Cites). Spectrometry is thus a non-destructive procedure, with great potential both for use on live birds and on museum specimens.

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References

- Andersson, S., Prager, M., Johansson, A., 2007. Carotenoid content and reflectance of yellow and red nuptial plumage in widowbirds (*Euplectes* spp.). *Funct. Ecol.* 21, 272–281.
- Bókony, V., Líker, A., Székely, T., Kis, J., 2003. Melanin-based plumage coloration and flight displays in plovers and allies. *Proc. R. Soc. Lond., B* 270, 2491–2497.
- Conover, W.J., Iman, R.L., 1981. Rank transformations as a bridge between parametric and nonparametric statistics. *Am. Stat.* 35, 124–129.
- Del Hoyo, J., Elliot, A., Christie, D.A. (Eds.), 2004. *Handbook of the Birds of the World*, vols. 4–9. Lynx Edicions, Barcelona.
- Fernández, A.A., Morris, M.R., 2007. Sexual selection and trichromatic color vision in primates: statistical support for the pre-existing-bias hypothesis. *Am. Nat.* 170, 10–20.
- Fox, D.L., Hopkins, T.S., 1966. Comparative metabolic fractionation of carotenoids in three flamingo species. *Comp. Biochem. Physiol.* 17, 841–856.
- Fry, C.H., Keith, S. (Eds.), 2004. *The Birds of Africa*, vol. VII. Christopher Helen, London.
- Grande, J.M., Negro, J.J., Torres, M.J., 2004. The evolution of bird plumage colouration: a role for feather degrading bacteria? *Ardeola* 51, 375–383.
- Gray, D.A., 1996. Carotenoids and sexual dichromatism in North American passerine birds. *Am. Nat.* 148, 453–480.
- Hill, G.E., 1991. Plumage coloration is a sexually selected indicator of male quality. *Nature* 350, 337–339.
- Hill, G.E., 1996. Redness as a measure of the production cost of ornamental coloration. *Ethol. Ecol. Evol.* 8, 157–175.
- Hill, G.E., 2006. Female mate choice for ornamental coloration. In: Hill, G.E., McGraw, K. (Eds.), *Bird Coloration*, vol. II. Function and Evolution. Harvard University Press, Cambridge MS, pp. 137–200.
- Hill, G.E., McGraw, K.J., 2003. Melanin, nutrition and the lion's mane. *Science* 299, 660.
- Hill, G.E., McGraw, K. (Eds.), 2006. *Bird Coloration*, Vols. I (Mechanisms and Measurements) and II (Function and Evolution). Harvard University Press, Cambridge MS.
- Margalida, A., Negro, J.J., Galván, I., 2008. Melanin-based color variation in the bearded vulture suggests a thermoregulatory function. *Comp. Biochem. Physiol., A* 149, 87–91.
- McGraw, K.J., 2006a. Mechanics of carotenoid-based coloration. In: Hill, G.E., McGraw, K. (Eds.), *Bird Coloration*, vol. I. Mechanisms and Measurements. Harvard University Press, Cambridge MS, pp. 177–242.
- McGraw, K.J., 2006b. Mechanics of uncommon colors: pterins, porphyrins, and psittacofulvins. In: Hill, G.E., McGraw, K. (Eds.), *Bird Coloration*, vol. I. Mechanisms and Measurements. Harvard University Press, Cambridge MS, pp. 354–398.
- McGraw, K.J., 2006c. Mechanics of melanin-based coloration. In: Hill, G.E., McGraw, K. (Eds.), *Bird Coloration*, vol. I. Mechanisms and Measurements. Harvard University Press, Cambridge MS, pp. 243–294.
- McGraw, K.J., Nogare, M.C., 2004. Carotenoid pigments and the selectivity of psittacofulvin-based coloration systems in parrots. *Comp. Biochem. Physiol., B* 138, 229–233.
- McGraw, K.J., Nogare, M.C., 2005. Distribution of unique red feather pigments in parrots. *Biol. Lett.* 1, 38–43.
- McGraw, K.J., Wakamatsu, K., Ito, S., Nolan, P.M., Jouventin, P., Dobson, F.S., Austic, R.E., Safran, R.J., Siefferman, L.M., Hill, G.E., Parker, R.S., 2004a. You can't judge a pigment by its color: carotenoid and melanin content of yellow and brown feathers in swallows, bluebirds, penguins, and domestic chickens. *Condor* 106, 390–395.
- McGraw, K.J., Safran, R.J., Evans, M.R., Wakamatsu, K., 2004b. European barn swallows use melanin pigments to color their feathers brown. *Behav. Ecol.* 15, 889–891.
- Mínguez-Mosquera, M.I., Hornero-Méndez, D., 1993. Separation and quantification of the carotenoid pigments in red peppers (*Capsicum annuum* L.), paprika and oleoresin by reversed-phase HPLC. *J. Agric. Food Chem.* 41, 1616–1620.
- Montgomerie, R., 2006a. Cosmetic and adventitious colors. In: Hill, G.E., McGraw, K. (Eds.), *Bird Coloration*, vol. I. Mechanisms and Measurements. Harvard University Press, Cambridge MS, pp. 399–427.
- Montgomerie, R., 2006b. Analyzing colors. In: Hill, G.E., McGraw, K. (Eds.), *Bird Coloration*, vol. I. Mechanisms and Measurements. Harvard University Press, Cambridge MS, pp. 90–147.
- Mullen, P., Pohland, G., 2008. Studies on UV reflection in feathers of some 1000 bird species: are UV peaks in feathers correlated with violet-sensitive and ultraviolet-sensitive cones? *Ibis* 150, 59–68.
- Negro, J.J., Margalida, A., 2000. How Bearded Vultures (*Gypaetus barbatus*) acquire their orange coloration: a comment on Xirouchakis (1998). *J. Raptor Res.* 34, 62–63.
- Negro, J.J., Margalida, A., Hiraldo, F., Heredia, R., 1999. The function of the cosmetic coloration of bearded vultures: when art imitates life. *Anim. Behav.* 58, F14–F17.
- Negro, J.J., Sarasola, J.H., Fariñas, F., Zorrilla, I., 2005. Function and occurrence of facial flushing in birds. *Comp. Biochem. Physiol., A* 143, 78–84.
- Piersma, T., Jukema, J., 1993. Red breasts as honest signals of migratory quality in a long-distance migrant, the bar-tailed godwit. *Condor* 95, 163–177.
- Pohland, G., Mullen, P., 2006. Preservation agents influence UV-coloration of plumage in museum bird skins. *J. Ornithol.* 147, 464–467.

- Prum, R.O., 2006. Anatomy, physics and evolution of structural colors. In: Hill, G.E., McGraw, K. (Eds.), *Bird Coloration*, vol. I. Mechanisms and Measurements. Harvard University Press, Cambridge MS, pp. 259–353.
- Pryke, S.R., Griffith, S.C., 2006. Red dominates black: agonistic signalling among head morphs in the colour polymorphic Gouldian finch. *Proc. R. Soc. Lond.*, B 273, 949–957.
- Ridgely, R.S., Tudor, G., 1994. *The Birds of South America*. Academy of Natural Sciences of Philadelphia. Oxford University Press. Oxford.
- Rimington, C., 1939. A reinvestigation of turacin, the copper porphyrin pigment of certain birds belonging to the Musophagidae. *Proc. R. Soc. Lond.*, B 127 (846), 106–120.
- Saino, N., Stradi, R., Ninni, P., Møller, A.P., 1999. Carotenoid plasma concentration, immune profile and plumage ornamentation of male Barn Swallows (*Hirundo rustica*). *Am. Nat.* 154, 441–448.
- Stradi, R., 1998. *The Colour of Flight: Carotenoids in Bird Plumage*. Solei Gruppo Editoriale Informatico, Milan.
- Stradi, R., Celentano, G., Rossi, E., Rovati, G., Pastore, M., 1995. Carotenoids in bird plumage 1. The carotenoid pattern in a series of Palearctic Carduelinae. *Comp. Biochem. Physiol.*, B 110, 131–143.
- Stradi, R., Rossi, E., Celentano, G., Bellardi, B., 1996. Carotenoids in bird plumage: the pattern in three *Loxia* species and in *Pinicola eunucleator*. *Comp. Biochem. Physiol.*, B 113, 427–432.
- Stradi, R., Celentano, G., Boles, M., Mercato, F., 1997. Carotenoids in bird plumage: the pattern in a series of red-pigmented Carduelinae. *Comp. Biochem. Physiol.*, B 117, 85–91.
- Stradi, R., Hudon, J., Celentano, G., Pini, E., 1998. Carotenoids in bird plumage: the complement of yellow and red pigments in true woodpeckers (Picinae). *Comp. Biochem. Physiol.*, B 120, 223–230.
- Stradi, R., Pini, E., Celentano, G., 2001. Carotenoids in bird plumage: the complement of red pigments in the plumage of wild and captive bullfinch (*Pyrrhula pyrrhula*). *Comp. Biochem. Physiol.*, B 128, 529–535.
- Svensson, L., Grant, P.J., Mullarney, K., Zetterstrom, D., 2000. *Collins Bird Guide*. Collins, London.
- Tabachnick, B.G., Fidell, L.S., 2001. *Using multivariate statistics*, Chapter 11: Discriminant Function Analysis, 4th Ed., pp. 456–491.
- Tella, J.L., Figuerola, J., Negro, J.J., Blanco, G., Rodríguez-Estrella, R., Forero, M.G., Blázquez, M.C., Green, A.J., Hiraldo, F., 2004. Ecological, morphological and phylogenetic correlates of interspecific variation in plasma carotenoid concentration in birds. *J. Evol. Biol.* 17, 156–164.
- Trams, E.G., 1969. Carotenoid transport in plasma of scarlet ibis (*Eudocimus ruber*). *Comp. Biochem. Physiol.* 28, 1177–1184.
- Westneat, D.F., 2006. No evidence of current sexual selection on sexually dimorphic traits in a bird with high variance in mating success. *Am. Nat.* 167, E171–E189.