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Porphyrins and pheomelanins contribute to the reddish juvenal plumage of black-shouldered kites

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ABSTRACT

Porphyrins are a widespread group of pigments in nature, but, contrary to melanins and carotenoids, their occurrence as plumage colorants seems to be anecdotal and their function, if any, is unknown. Using thin-layer chromatography and high pressure liquid chromatography, we have found coproporphyrin III, the same porphyrin type previously reported in owls, in the plumage of nestling black-shouldered kites (*Elanus caeruleus*). The first plumage grown at the nest in this species includes reddish-brown contour feathers in the upperparts, and particularly in the breast area, which fade during the weeks-long post-fledging period to become either gray or white consistent with the definitive adult plumage. In these reddish feathers, we have also found small amounts of pheomelanins and traces of eumelanin. The contribution of each pigment to the final colour perceived by birds or other animals is unknown. In white and grey feathers of the same species no porphyrin was found, and only traces of eumelanin were detected in the grey ones. The fact that the reddish feathers are only found in the juvenal plumage, when individuals are vulnerable in an open nest, leads us to hypothesize a camouflage role for this ephemeral plumage. As porphyrins are involved, although not exclusively, we can for the first time ascribe them a function in the plumage of birds.

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1. Introduction

Porphyrins are a widespread group of pigments in nature, including chlorophyll for photosynthesis in plants and hemoglobin for oxygen transportation in the blood of vertebrates. However, and contrary to the two other major pigment types that evolved early in the history of life, i.e., melanins and carotenoids, our knowledge of the occurrence of porphyrins as plumage colorants seems to be largely anecdotal (McGraw, 2006a). Porphyrins are known to occur in 13 avian orders, but are said to be practically restricted to the plumage of owls (Order Strigiformes), goatsuckers (Order Caprimulgiformes) and bustards (Order Gruiformes (With, 1978). Feather porphyrins confer a reddish-brown colour, not very different in terms of both apparent coloration to the human eye and spectral reflectance properties to pheomelanin (Toral et al., 2008).

In fresh or emerging feathers, porphyrins fluoresce and appear pink to humans when illuminated solely by ultra violet (UV) light (With, 1978). This fluorescence is, however, photo-degradable and quickly fades in light-exposed feathers (authors' unpubl. obs.). Plumage porphyrins are not only intriguing for their fluorescent properties and comparative rarity. No function has yet been ascribed to them, although

a thermoregulatory role has been suggested because porphyrins do not absorb infrared light (McGraw, 2006a).

Here we describe the presence of one particular type of porphyrin, i.e. coproporphyrin III, in the plumage of nestling black-shouldered kites (*Elanus caeruleus*). The first plumage grown at the nest in this species includes reddish-brown contour feathers in the upperparts, from head to tail, and particularly in the breast area, which fade to become either gray (in the upperparts) or white (in the breast), consistent with the definitive adult plumage (Fergusson-Lees and Christie, 2001), during the several weeks-long post-fledging dependence period (Bustamante, 1993). The fact the reddish feathers are only found in the juvenal plumage, and given that the nestlings are raised in open nests located at tree tops, where they are susceptible to predation by larger and visually oriented birds of prey, leads us to hypothesize a camouflage role for this plumage in this species. This protective function is typically achieved in other species by the intricate deposition of melanins in the plumage (Bortolotti, 2006).

2. Material and methods

2.1. Study species

The four extant species in *Elanus* are small raptorial birds showing ecological, behavioural and morphological adaptations intermediate between the diurnal raptors and the owls (Negro et al., 2006). These

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kites are specialists in hunting cyclic or irruptive small rodents and, in the southern hemisphere they appear to fill the niche of the nomadic owls of the northern hemisphere. As with owls, *Elanus* kites have zygodactyl feet and soft-barbed feathers for silent flight.

All species of *Elanus* are remarkably similar in plumage colour and size – about 300 g adult mass. *E. caeruleus* from southern Asia and Africa, *E. leucurus* from the Americas and *E. axillaris* from Australia are so similar in appearance and behaviour that at one time they were considered a cosmopolitan super-species (Parkes, 1958). In all four species, the juvenal plumage consists of reddish-brown feathers in the upperparts and breast (Fergusson-Lees and Christie, 2001), whereas the adult plumage is white and grey with small black patches. Here, we analysed pigment content of the black-shouldered kite, *E. caeruleus*, of the Iberian peninsula.

2.2. Feather origin

Feather samples with the reddish-brown pigment, as well as white or grey coloured feathers, were obtained from two nestlings found depredated at the nest in 2007. Both originated from nests in southern Spain, one in the province of Huelva and the other in the province of Badajoz. The collected feathers did not fluoresce perceptibly under UV light in the laboratory. As a control, we used feathers of a tawny owl (*Strix aluco*) deposited at the Vertebrate Collection of Doñana Biological Station (Sevilla, Spain). Even though the specimen had been prepared years before, it still showed pink fluorescence in the underwings, and this is the area we sampled for analysis. The tawny owl may be used as a positive control because this species was reported to have coproporphyrin III in a previous study (With, 1978).

2.3. Pigment identification

2.3.1. Extraction and thin layer chromatography

After having observed that the reddish colour of the black-shouldered kite feather faded with time, we suspected the presence of porphyrins and, as a first approach to detect them, we used thin layer chromatography (TLC) following With (1978). In addition to the reddish feathers, we also extracted grey and white kite feathers. As controls, we used tawny owl secondary feathers, as well as standard coproporphyrin and protoporphyrin purchased from Frontier Scientific Ltd. (Carnforth, UK). Following With (1978), we weighed 20–140 mg of feathers of both kites and owls, and were extracted in 80% acetic acid in the dark at room temperature for a week. The extract was separated from the feathers by decantation with two volumes of ethyl acetate and distilled water. The aqueous phase was extracted once more with ethyl acetate and the combined organic phases were washed with 3% sodium acetate, and finally the porphyrins were transferred to 1 M HCl. The extracts and the controls were subjected to TLC on silica gel plates (Alufolien-Silica Gel 60 F₂₅₄, Merck) and to HPLC analysis.

To determine the presence of melanins, we re-extracted the reddish, grey and white feathers previously subjected to the porphyrin extraction protocol with NaOH (Toral et al., 2008), and centrifuged the solution to get soluble pheomelanin (brownish in appearance) in the supernatant and a black pellet containing eumelanin. The relative concentration (absorbance units/feather mass) of both melanin types was estimated spectrophotometrically at 450 nm as in Toral et al. (2008).

2.3.2. HPLC analysis

Both black-shouldered kite and tawny owl feathers were analyzed by HPLC following a protocol modified from Mateo et al. (2004), and with fluorescence detection. A HP1100 series quaternary pump, autosampler, column oven and diode array detector were used (Seeltze, Germany). All the chromatographic conditions and quantification were controlled using ChemStation software (ver. A.06.01).

A Waters (Milford, MA, USA) Spherisorb ODS 2 (5 µm particle size, 4.6 mm × 100 mm) chromatographic column was used. The flow rate was 1.5 ml/min and a solvent gradient was used. The initial mobile phase composition was methanol 25% and ammonium acetate (1.0 M, pH 5.16) 75% for 2 min. The solvent gradient consisted in an 8 min linear change to 95% methanol and 5% ammonium acetate, followed by 2 min at these conditions. At this moment the phase composition returned to the initial conditions in 3 min and remained at this status for 3 min. The total run time was 18 min. The column was maintained at 70 °C and the detection was with excitation wavelength of 400 nm and emission wavelength of 620 nm. Standard coproporphyrin III (4-carboxyl porphyrin) was purchased from Frontier Scientific Ltd. (Carnforth, UK).

3. Results

As expected of porphyrins, the reddish kite samples and the owl ones run in TLC plates fluoresced under UV light. This alone confirmed that the extracts obtained from the reddish feathers of the juvenal plumage of the black-shouldered kite contained a type of porphyrin, although it remained unidentified. The subsequent HPLC analysis singled out coproporphyrin III by comparison with commercial standards. The resulting chromatograms for the kite, owl and coproporphyrin III standard all showed a single peak eluting at 7.4 min (Fig. 1). Melanin results are given in Table 1. Pheomelanin concentration averaged 0.116 Absorbance units/mg of feather (Au/mg), compared to 0.033 Au/mg in grey feathers and 0.016 in white feathers. The latter can be considered a trace level, as with eumelanin estimations in the range 0.01–0.02 (Au/mg) in all three types of feathers (see Table 1). The amount of pheomelanin in the reddish feathers was the lowest compared to that of other bird species with red or rusty feathers analysed in our laboratory (see, Toral et al., 2008). As an example, the

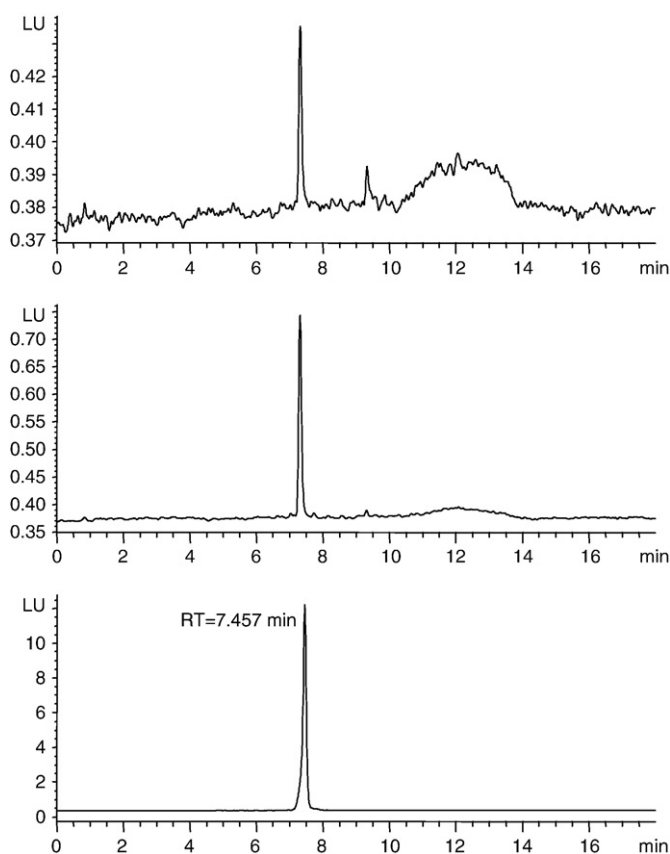


Fig. 1. HPLC chromatograms for a black-shouldered kite (top), tawny owl (center) and coproporphyrin III standard (bottom).

Table 1

Relative concentration values expressed as absorbance units/mg feather (Au/mg) of pheomelanin and eumelanin in three different feathers coloured red, grey and white of a juvenile black-shouldered kite (*Elanus caeruleus*).

Feather colour	Pheomelanin (Au/mg)	Eumelanin (Au/mg)
Red 1	0.11	0.02
Red 2	0.13	0.02
Red 3	0.11	0.06
Mean red	0.116	0.03
Grey 1	0.04	0.02
Grey 2	0.03	0.02
Grey 3	0.03	0.09
Mean grey	0.033	0.043
White 1	0.01	0.01
White 2	0.01	0.01
White 3	0.03	0.04
Mean white	0.016	0.02

Mean values are also given.

red feathers in the throat of the swallow (*Hirundo rustica*) averaged 0.49 Au/mg of pheomelanin, or four times the concentration in the reddish feathers of black-shouldered kites.

4. Discussion

This is the first time a porphyrin has been identified in the plumage of a diurnal raptor (Order Falconiformes). As stated above, plumage porphyrins are thought to be typical of owls, goatsuckers and bustards (McGraw, 2006a). Curiously enough, a number of evolutionary convergences have recently been reported between the black-shouldered kites and owls (Negro et al., 2006). However, the convergent deposition of porphyrins in the plumage was previously unknown. The function of pigments in the plumage of birds has been widely debated: carotenoids are said to signal condition and/or foraging efficiency (McGraw, 2006b, and references therein), whereas melanins tend to provide crypsis and abrasion resistance, while also being involved in mate attraction (McGraw, 2006c), as well as dominance signalling or nest defence (Senar, 2006). To date, no function has been ascribed to the porphyrins found in feathers (McGraw, 2006a). In the case of *Elanus*, porphyrins may have no function and are solely an excretion product of the metabolism of the red cells; however, why should such a trait only occur in reddish feathers of nestlings? We here hypothesize that the porphyrins along with pheomelanin may confer crypsis at a time in the life cycle when individuals are most vulnerable, i.e., when they are confined to the nest site and are incapable of escaping or defending themselves when attacked by a predator.

Delayed plumage maturation (DPM) with a cryptic function has been suggested for numerous bird species (e.g., Selander, 1965; Procter-Gray and Holmes, 1981; Hill, 1988; Stutchbury, 1991), but the potential cryptic function of DPM has not been fully recognized because juvenile birds are typically subjected to many additional selective forces, including intraspecific aggression avoidance during their first winter (Senar, 2006). The fact that porphyrins degrade, even though pheomelanins do not, as soon as they are exposed to ambient light, provides support for the first time to the mechanism of DPM with a cryptic function.

4.1. Camouflage

Like many other birds, raptors often possess a juvenal plumage that is cryptic by comparison with the definitive feathering of their parents (e.g., *Accipiter* spp.), or for sexually dichromatic species similar to the less colourful plumage of females (e.g., *Circus* spp., most kestrels (*Falco* spp.)). The cryptic plumage of the incubating sex has generally been viewed as an adaptation for concealment at the nest (Edelstam, 2001), and so by inference should the plumage of nestlings. Crypsis may be advantageous for young *Elanus* sp. for protection against a

plethora of much larger diurnal raptors. In a study we carried out in 2004–2005 in Spain (see, e.g., Balbontín et al., 2008), depredation at the nest accounted for 42% of nestling mortality ($n=51$ dead nestlings in total), followed by starvation (31%). Depredation was again the main mortality factor for adult birds in our study area (32% of 31 birds that died). In the Iberian peninsula alone there are perhaps 10 or more sympatric raptor species capable of preying on young kites, including the booted eagle (*Hieraetus pennatus*), the peregrine falcon (*Falco peregrinus*), the common buzzard (*Buteo buteo*) and the eagle owl (*Bubo bubo*). There are considerably more within the species' range in Africa, and Mendelsohn (1983) reported predation by *Aquila rapax*, *Bubo africanus* and *Falco peregrinus*. The Iberian population of *E. caeruleus* has very specific habitat and nesting requirements. They almost exclusively nest in oak (*Quercus* spp.) dehesa (open savannah) habitat (Balbontín et al., 2008). As their platform nests are situated at the tops of trees, white offspring would be both conspicuous and accessible.

4.2. Why porphyrin and melanins in the same feathers?

These two pigment types were also found concurrently in previously studied bustards and owls (With, 1978), but no function was then ascribed to the porphyrins, and it was assumed that the melanins were entirely responsible for the visible or perceptible colour of the feathers. In the case of the reddish juvenile plumage of the black-shouldered kite, we have stated above that it seems to provide crypsis when it is most needed. The contribution to feather coloration of each of the pigments involved is still unclear to us. The amount of pheomelanin is low compared to levels in other species with red or yellowish plumages (data not shown), and the plumage actually fades rapidly as it is expected of the photodegradable porphyrins. These two facts suggest that the two pigments acting together indeed facilitate the reported effect: transient camouflage through the use of an inconspicuous plumage different to the definitive one present in the adult birds. More research is however needed to determine the proportional contribution of both porphyrin and pheomelanin. This finding also warrants further investigations regarding the actual prevalence of porphyrins in the plumage of birds, which could be much higher than currently assumed.

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References

- Balbontín, J., Negro, J.J., Sarasola, J.H., Ferrero, J.J., Rivera, D., 2008. Land-use changes may explain the recent range expansion of the black-shouldered kite (*Elanus caeruleus*) in southern Europe. *Ibis* 150, 707–716.
- Bortolotti, G., 2006. Natural selection and coloration: protection, concealment, advertisement or deception? In: Hill, G.E., McGraw, K. (Eds.), *Bird Coloration*, vol. II. Function and Evolution. Harvard University Press, Cambridge MS, pp. 3–33.
- Bustamante, J., 1993. The post-fledging dependence period of the black-shouldered kite (*Elanus caeruleus*). *J. Raptor Res.* 27, 185–190.
- Edelstam, C., 2001. Raptor plumages and external structure. In: Fergusson-Lees, J., Christie, D.A. (Eds.), *Raptors: Birds of Prey of the World*. A & C Black Pub. Ltd., London, pp. 57–69.
- Fergusson-Lees, J., Christie, D.A., 2001. *Raptors: Birds of Prey of the World*. A & C Black Pub. Ltd., London.
- Hill, G.E., 1988. The function of delayed plumage maturation in male black-headed grosbeaks. *Auk* 105, 1–10.
- Mateo, R., Castell, G., Green, A.J., Godoy, C., Cristofol, C., 2004. Determination of porphyrins and biliverdin in bile and excreta of birds by a single liquid chromatography – ultraviolet detection analysis. *J. Chromatogr. B* 810, 305–311.
- McGraw, K.J., 2006a. 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., 2006b. 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., 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.
- Mendelsohn, J., 1983. Causes of mortality in black-shouldered kites. *Bokmakierie* 35, 11–13.
- Negro, J.J., Pertoldi, C., Randi, E., Ferrero, J., López-Caballero, J., Rivera, D., Korpimäki, E., 2006. Convergent evolution of elanus kites and the owls. *J. Raptor Res.* 40, 222–225.
- Parkes, K., 1958. Specific relationships in the genus *Elanus*. *Condor* 60, 139–140.
- Procter-Gray, E., Holmes, R.T., 1981. Adaptive significance of delayed attainment of plumage in male american redstarts: tests of two hypotheses. *Evolution* 35, 742–751.
- Selander, R.K., 1965. On mating systems and sexual selection. *Am. Nat.* 99, 129–141.
- Senar, J.C., 2006. Color displays as intrasexual signals of aggression and dominance. In: Hill, G.E., McGraw, K.J. (Eds.), *Bird Coloration*, Volume 2: function and evolution. Harvard University Press, Cambridge, MA.
- Stutchbury, B.J., 1991. The adaptive significance of male subadult plumage in purple martins: plumage dyeing experiments. *Behav. Ecol. Sociobiol.* 29, 297–306.
- Toral, G.M., Figuerola, J., Negro, J.J., 2008. Multiple ways to become red: pigment identification in red feathers using spectrometry. *Comp. Biochem. Physiol. A* 150, 147–152.
- With, T.K., 1978. On porphyrins in feathers of owls and bustards. *Int. J. Biochem.* 9, 893–895.