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**ORIGINAL ARTICLE**

# Feather content of porphyrins in Eurasian eagle owl (*Bubo bubo*) fledglings depends on body condition and breeding site quality

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## Abstract

Porphyrins are pigments produced in most animal cells during the synthesis of heme, but their importance for external coloration is unclear. Owls (Order Strigiformes) are among the few animals that accumulate porphyrins in the integument, where it could serve as a means of signaling. Here we hypothesized that the porphyrin content of feathers may depend on body condition and breeding site quality in Eurasian eagle owl (*Bubo bubo*) fledglings and, thus, constitute amplifiers of the quality of the area where they are born. Using high-performance liquid chromatography, we found 2 porphyrins (protoporphyrin IX and coproporphyrin III) in the body feathers of 19 eagle owl fledglings from 7 breeding territories. Coproporphyrin III, but not protoporphyrin IX feather concentration, was positively associated with the body mass of fledglings and with the quality of the breeding sites where they were reared with respect to food quality and availability. As coproporphyrin III is produced under oxidative stress, we suggest that good breeding sites may lead to fledglings in good condition. This, in turn, may make fledglings induce a certain level of free radical and coproporphyrin III production to signal to conspecifics their site-mediated capacity to cope with oxidative stress. This is the first time that porphyrin content in the integument has been found to be related to individual quality, opening a new scenario for studying evolution of animal coloration.

**Key words:** amplifiers, condition-dependence, eagle owl, fluorescence, porphyrins

## INTRODUCTION

Animal coloration fulfills a diversity of functions, like protection against environmental agents and visual communication (Burtt 1981). The latter includes signaling individual attributes, such as genetic quality to conspecifics (e.g. parents or potential mates), a function whose understanding critically depends on the mechanisms that lead to color production (Galván *et al.* 2015).

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Most animal colors are produced by the deposition of pigments in the skin and associated appendages such as hairs, feathers and scales. It is, thus, essential to know which factors affect the synthesis of these pigments to determine the information content of animal coloration.

There is a large diversity of biological pigments, but they can be grouped into just 3 common metabolic routes (Gudin 2003). The synthesis of the most abundant animal pigments, termed melanins, follows the shikimic route. This pathway depends on the common precursor shikimic acid, from which microorganisms and plants synthesize phenylalanine, an essential amino acid for animals. Animals convert phenylalanine into tyrosine, the basic precursor of melanins (d'Ischia *et al*. 2015). The mevalonic route, in contrast, leads to the production of the second main group of animal pigments (i.e. carotenoids) responsible for most of the brightest colors which are synthesized mainly by plants with the mevalonic acid as a common precursor. Finally, a third route termed levulinic leads to the production of the pigments porphyrins, using the levulinic acid as a common precursor (Gudin 2003). Porphyrins may be the most abundant biological pigments as this group includes molecules that are key for life, such as chlorophyll and heme, but their occurrence in animals out of internal tissues and fluids is known to be limited to only certain fishes, the European hedgehog (*Erinaceus europaeus* Linnaeus, 1758) and anomalous depositions in the human skin during certain diseases (porphyrias) (McGraw 2006). Thus, the levulinic route is currently believed not to be significant for the evolution of animal visual communication.

Apart from the examples mentioned above, birds constitute another exception for the animal levulinic route, as some groups, most notably owls (Order Strigiformes), nightjars (Order Caprimulgiformes) and bustards (Order Otidiformes), have evolved a physiological capacity to deposit porphyrins in feathers (Völker 1938). The presence of porphyrins in the integument of birds from different orders does not necessarily support the idea that the levulinic route contribute to animal color diversity, as despite porphyrins exhibiting fluorescent properties (i.e. emit long-wavelength light when excited by ultraviolet [UV] light), the perceptible color that these pigments generate in the animal integument without artificial UV light excitation has been assumed to be negligible at least for humans (Negro *et al*. 2009). However, Galván *et al*. (2016) demonstrate that porphyrins actually create very conspicuous salmon-pink coloration in the feathers of bustards. This conspicuous coloration had remained unnoticed likely because the color disappears after a few minutes of exposure to sunlight. This intense color results from the highly conjugated structure of porphyrins, which are composed of 4 pyrroles connected by methine bridges to form aromatic rings (Fig. 1) that open when exposed to visible light and, in turn, lead to the photodegradation of the pigment (Rotomskis *et al*. 1996). Therefore, porphyrins have the potential to produce conspicuous colorations in birds that may evolve as visual signals (Galván *et al*. 2016).

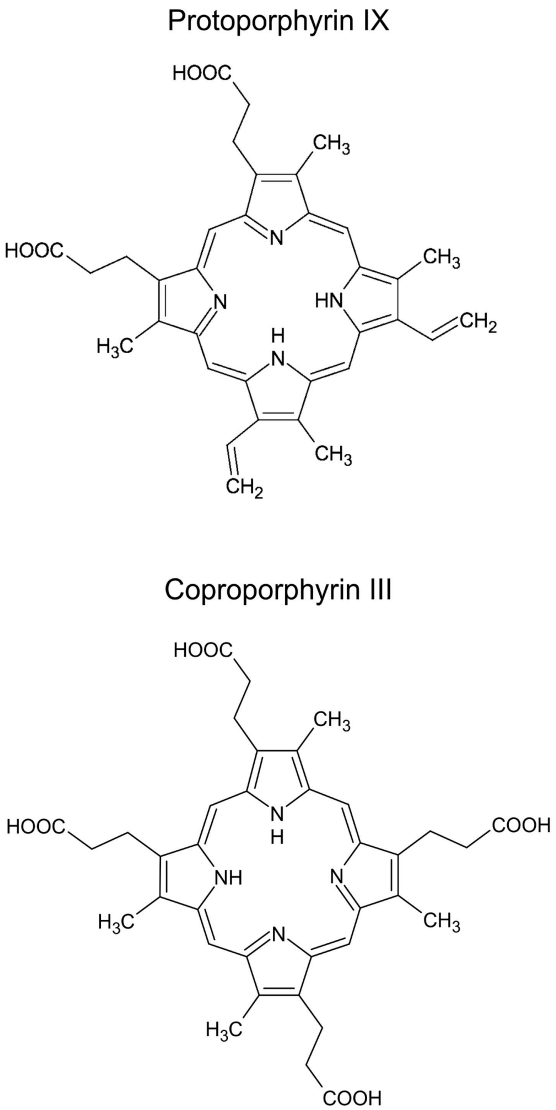


**Figure 1** Chemical structure of protoporphyrin IX and coproporphyrin III.

Currently, there is little knowledge about the information that birds may transfer to conspecifics through porphyrin-based signals. It has been suggested that they may be amplifiers of the reproductive status of male bustards, as any female that finds porphyrin-based, salmon-pink coloration in a displaying male may determine that the male has not displayed for a long period of time (as the color is only exposed during displays and progressively photodegrades) and, thus, obtain higher quality sperm (which decreases over successive copulations) (Galván *et al.* 2016). However, this explanation may only be valid for mating strategies such as leks in which males copulate with multiple females within a breeding season. Thus, additional information is needed for the other groups of birds known to deposit porphyrins in feathers that do not form leks (i.e. owls and at least some nightjars).

Our aim here is to investigate the signaling potential for porphyrins in the feathers of Eurasian eagle owl (*Bubo bubo* Linnaeus, 1758) fledglings. The salmon-pink coloration that is found in bustard feathers not exposed to sunlight has never been observed in owls, and, thus, the deposition of porphyrins in the feathers of eagle owls may be unique to that species. However, we cannot completely rule out the possibility that a conspicuous coloration is produced in the owls' feathers only during a brief period of time before the pigment photodegrades as in bustards (Galván *et al.* 2016). Furthermore, and despite that the fluorescence of porphyrins in owls' feathers is only perceptible to humans after excitation with artificial UV light (Weidensaul *et al.* 2011), the possibility that owls can actually perceive the fluorescence of porphyrins under natural light should not be discarded either. Porphyrin-based coloration in the feathers of owls is not likely to evolve as an amplifier of reproductive status as in bustards (see above), but it may have a role in sexual selection by acting as an amplifier of breeding site quality if the development of fledglings, including the production of porphyrins, depends on nutritional resources and, thus, on the quality of the breeding site where they are reared (e.g. Wolfenbarger *et al.* 1999; Galván *et al.* 2009). To test this hypothesis, we predicted that site quality should be positively associated with the content of porphyrins in feathers, which, in turn, should be positively associated with the body condition of fledglings. This is the first investigation of possible relationships between individual attributes and porphyrins content in the animal integument.

A second alternative is that porphyrin-based coloration in eagle owls has a role in sexual selection by acting as a handicap signal; that is, a signal whose expression is limited to individuals of high genetic quality because of its production or maintenance costs (Hasson 1997). In this case, there should be a positive association between the porphyrin content of feathers and the body condition of birds because handicap signals are expected to show heightened condition-dependence (Cotton *et al.* 2004), but there should be no environmental lability and, hence, no association with breeding site quality. This is because porphyrins are synthesized by animals and, to date, no metabolic costs are known for these pigments; thus, there should be no trade-offs between using porphyrins for pigmentation and for health as in other pigments that act as handicap signals and must be taken in the diet (i.e. carotenoids; Blount *et al.* 2003).

Although sexual selection only operates in adults, the expression of a porphyrin-based trait in fledglings could result from the expression of the genes that control the development of the trait in all age classes (Johnsen *et al.* 2003). Moreover, porphyrin-based coloration may be involved in signaling genetic quality to parents instead of potential mates (e.g. Galván *et al.* 2008; Romano *et al.* 2016), which also predicts a positive association between the porphyrin content of feathers and the body condition of fledglings. Indeed, eagle owl fledglings seem to visually communicate their physical condition to their parents by the brightness of the white feathers surrounding their mouths (Penteriani *et al.* 2007).

## MATERIALS AND METHODS

### Study area and animals

The study was carried out in 2014 during the nestling season (March–April in our study area) in a population of eagle owls in Sierra Norte de Sevilla (Sierra Morena, southwest Spain; e.g. Delgado *et al.* 2013; Lourenço *et al.* 2015). We visited 7 breeding sites, located the nests and determined the laying date by estimating the age of fledglings following Penteriani *et al.* (2005). A total of 19 fledglings ranging from 18 to 37 days from 7 nests were included in the study (Fig. 2a). We measured the body mass of birds to the nearest 10 g using a Pesola balance, excepting 2 fledglings in which body mass could not be measured for logistical reasons. Body mass alone is a good body condition index (i.e. reflects fat content) in birds (Labocha & Hayes 2012).

We plucked 2–4 body feathers from each fledgling and stored them in dark envelopes until the chemical

analyses were conducted. As porphyrins are photolabile pigments, a single measurement of porphyrin content in feathers may not be representative of the average content for a given bird if the level of past exposure to sunlight is not controlled for, which is usually impractical in wild population studies. Therefore, every fledgling was sampled for feathers 2 times (9 fledglings) or 3 times (5 fledglings) on a weekly basis until fledging, excepting 5 fledglings from 2 different nests that could be sampled only once. Only feathers, not downy, were collected, and, thus, eagle owl fledglings were not sampled until they had developed contour body feathers.


**Figure 2** Photographs of eagle owl fledglings and feathers. (a) Eagle owl fledglings from one of the breeding sites included in the study. (b) Red fluorescence produced by porphyrins during illumination with ultraviolet light in 1 feather of 1 of the fledglings. Photo credits: Juan José Negro.

To localize the feathers that contain porphyrins, we covered the fledglings with a dark blanket and examined their body (avoiding the head) using an Opti-Lux OLX-365 LED lamp (Spectronics Corporation, Westbury, USA) with maximum emission at 365 nm, thus allowing the excitation of porphyrins, which then emit light at visible light (Karolczak *et al.* 2004). In birds, porphyrins produce intense red fluorescence in the base of feathers (Galván *et al.* 2016) (Fig. 2b); thus, we collected the feathers exhibiting the most intense red color from eagle owl fledglings irrespective of body regions.

## Breeding site quality

Following Delgado *et al.* (2013) and Lourenço *et al.* (2015), we described the quality of the breeding site by means of: (ii) 2 measures of productivity (Penteriani *et al.* 2004), the mean and the coefficient of variation of young fledged per breeding pair; and (ii) the rabbit biomass contribution to eagle owl diet obtained for the 7 breeding sites by previous diet analyses and through the collection of a minimum of 100 pellets for each nesting site (mean biomass percentage of rabbit in the diet ± SD: 63.3 ± 15.8%, range: 24.8%–93.7%; for more details see Lourenço *et al.* [2015]). Indeed, rabbits represent the main eagle owl prey in the study area (Campioni *et al.* 2013; Delgado *et al.* 2013; Lourenço *et al.* 2015) and, more generally, diet is related to crucial aspects of life-history and, ultimately, to individual fitness (Campioni *et al.* 2013; Lourenço *et al.* 2015). The rabbit biomass percentage was determined by analyzing prey remains and pellets collected at the end of the breeding season (April–June) from 2003 to 2008 during visits to nests and roosting/feeding posts. We pooled all samples from each breeding site and determined the minimum number of individuals of each species by counting the most frequent bone, generally long bones from front and hind limbs. We identified prey individuals using identification keys for bones and feathers and a reference collection (Laboratory of Archaeo-sciences, IGESPAR, Portugal). We calculated the rabbit biomass using its mean weight value from bibliographic references or bone measurements to estimate the weight of each individual. In exploratory analysis we found no annual differences in diet parameters within breeding sites; therefore, we pooled the data from all years to obtain a representative sample size for each breeding site (Lourenço *et al.* 2015).

## Analysis of porphyrins in feathers

Individual eagle owl feathers were separately an-

alyzed by high-performance liquid chromatography (HPLC) following a protocol modified from Mateo *et al.* (2004), and with fluorescence detection. Feathers were excised from the rachis, weighted (mean: 30.44 mg, range: 2.12–100.89 mg), trimmed and 0.83 mL of HCl 3N and 1 mL of acetonitrile were added. The mixture was shaken, incubated at dark for 30 min and then sonicated at cold for 15 min. Extractions were centrifuged at 10 000 *g* and 4 °C for 5 min, filtered and added to HPLC vials. An HP1200 series quaternary pump, autosampler, column oven and diode array detector were used (Seeltze, Germany). All chromatographic conditions and quantification were controlled using ChemStation software (ver. B.04.02). A Waters (Milford, MA, USA) Spherisorb ODS 2 (5-µm particle size, 4.6 mm × 100 mm) chromatographic column was used. The injection volume was 40 µL. The flow rate was 0.8 mL/min and a solvent gradient elution was used. The initial mobile phase composition was methanol 25% and ammonium acetate (10 mM, pH 5.16) 75% for 4 min. The solvent gradient consisted in a 20 min linear change to 100% methanol, followed by 2 min at these conditions. At this moment the phase composition returned to the initial conditions in 5 min and remained at this status for another 5 min. The total run time was 36 min. The column was maintained at 60 °C and detection by a fluorescence detector was made with excitation wavelength of 403 nm and emission wavelength of 603 nm, also including detection by a diode array detector at 402 nm, as this coincides with the maximum absorbance of both standard and extracted porphyrins from the eagle owl feathers: protoporphyrin IX (3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropanoic acid) and coproporphyrin III (3,8,13,17-tetramethylporphyrin-2,7,12,18-tetrapropanoic acid) (Figs 2 and 3). Standard porphyrins were purchased from Frontier Scientific (Carnforth, UK). Concentrations of porphyrins are expressed as pmol per gram of feather.

## Statistical analyses

Linear mixed-effects models (LMM) fit with restricted maximum likelihood (REML) estimation were used to analyze the associations between porphyrin concentration in feathers (response variable) and the body mass of fledglings (predictor variable), including nest and fledgling identity as random factors, employing SAS software and using the mixed procedure and the Satterthwaite method to calculate degrees of freedom. Marginal $R^2$ (i.e. variance explained by fixed terms only) and conditional $R^2$ (i.e. variance explained by both fixed and random terms) (Nakagawa & Schielzeth 2013) were calculated in R environment using the "piecewiseSEM" package. The difference between the corrected Akaike information criterion (AICc) (Burnham & Anderson 2002) value of the full models and the null models (i.e. those with the intercept only) was also calculated. The mean porphyrin concentration of all feathers collected from a given bird the same day was used in the analyses. Porphyrin concentration and body mass were

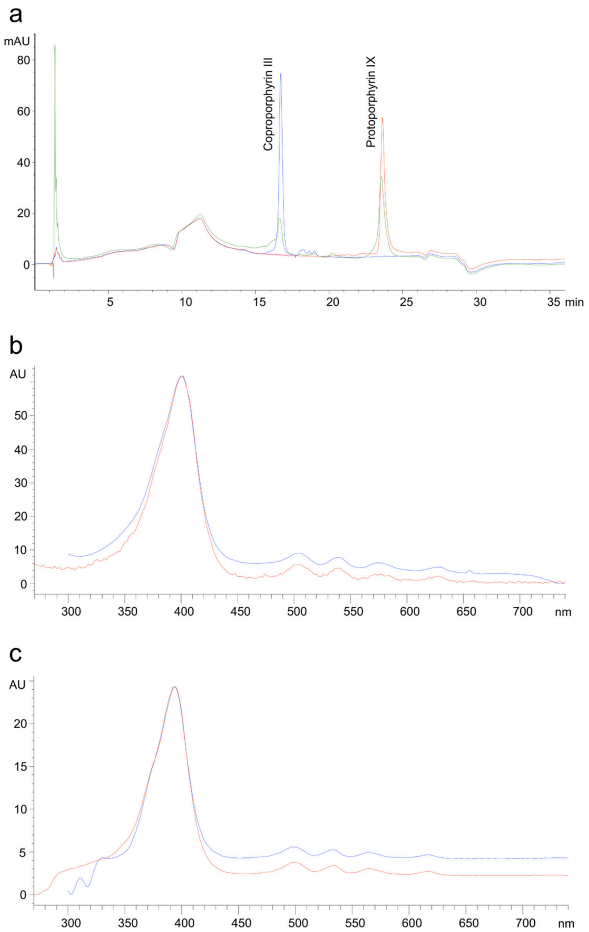


**Figure 3** Results of high-performance liquid chromatography analyses of porphyrins extracted from eagle-owl feathers. (a) Chromatogram of the diode array detector signal from an extraction of a feather (green curve) and from standards of protoporphyrin IX (red curve) and coproporphyrin III (blue curve). (b) Absorption spectrum of standard protoporphyrin IX (red curve) and natural protoporphyrin IX extracted from the same feather (blue curve). (c) Absorption spectrum of standard coproporphyrin III (red curve) and natural coproporphyrin III extracted from the same feather (blue curve).

$\log_{10}$-transformed to achieve normality assumptions, which were evaluated by inspection of residuals from the models. As breeding site characteristics are common to all fledglings from the same nests, we used the mean porphyrin concentration of fledglings in each nest and conducted Spearman rank correlation tests to analyze the association between porphyrin content in feathers and breeding site quality variables.

## RESULTS

Two porphyrins were detected in all feathers analyzed, protoporphyrin IX always prevailing (mean ± SE: 113.97 ± 10.90 pmol/g) on coproporphyrin III (33.14 ± 5.60 pmol/g). Fledgling body mass significantly and positively predicted the concentration of coproporphyrin III in feathers ($b = 2.93$, $F_{1,25.8} = 35.05$, $P < 0.0001$; Fig. 4), explaining 35.1% of variance in this variable. The effects of nest ($P = 0.155$) and fledgling identity ($P = 0.103$) were not significant (overall variance explained in coproporphyrin III concentration: 74.9 %; ΔAICc = 17.6). By contrast, fledgling body mass did not predict the concentration of protoporophyrin IX in feathers (2.3 % of variance; $b = 0.35$, $F_{1,32.0} = 0.91$, $P = 0.346$), the effects of nest ($P = 0.499$) and fledgling identity ($P = 0.357$) being equally non-significant (overall variance explained: 14.1 %; ΔAICc = 5.2). Simple correlation tests between mean body mass and mean porphyrin concentration for the 19 fledglings without controlling for the effect of nest identity confirmed the positive tendency for coproporphyrin III ($r = 0.35$, df = 17, $P = 0.141$), which yielded a much larger effect size than porphyrin IX ($r = -0.09$, df = 17, $P = 0.726$).

Considering the mean feather porphyrin concentration per nest, we found that coproporphyrin III concentration was also significantly and positively correlated with the coefficient of variation of productivity of the breeding sites ($r_s = 0.94$, $P < 0.005$; Fig. 5a) and with the biomass of rabbits in the diet of each breeding site ($r_s = 0.89$, $P < 0.05$; Fig. 5b) but not with the mean productivity ($r_s = -0.39$, $0.2 < P < 0.5$). By contrast, protoporphyrin IX concentration was not correlated with any index of breeding site quality (mean productivity: $r_s = -0.36$, $P > 0.5$; CV productivity: $r_s = 0.77$, $0.1 < P < 0.2$; rabbit biomass: $r_s = 0.71$, $0.1 < P < 0.2$).

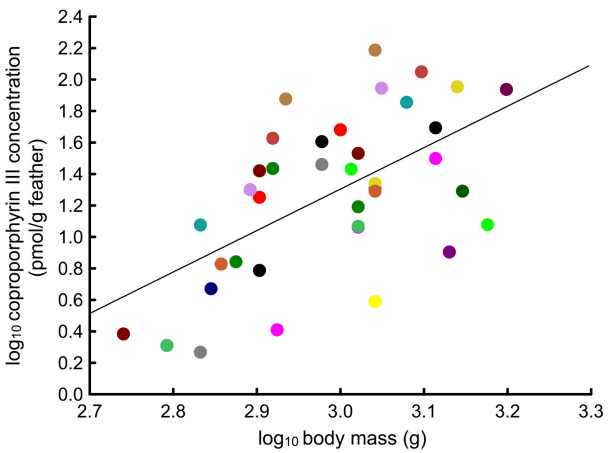


**Figure 4** Relationship between concentration of coproporphyrin III in feathers and body mass (which reflects body condition) of eagle-owl fledglings. Same color symbols correspond to the same individual bird. The best-fit line is shown.

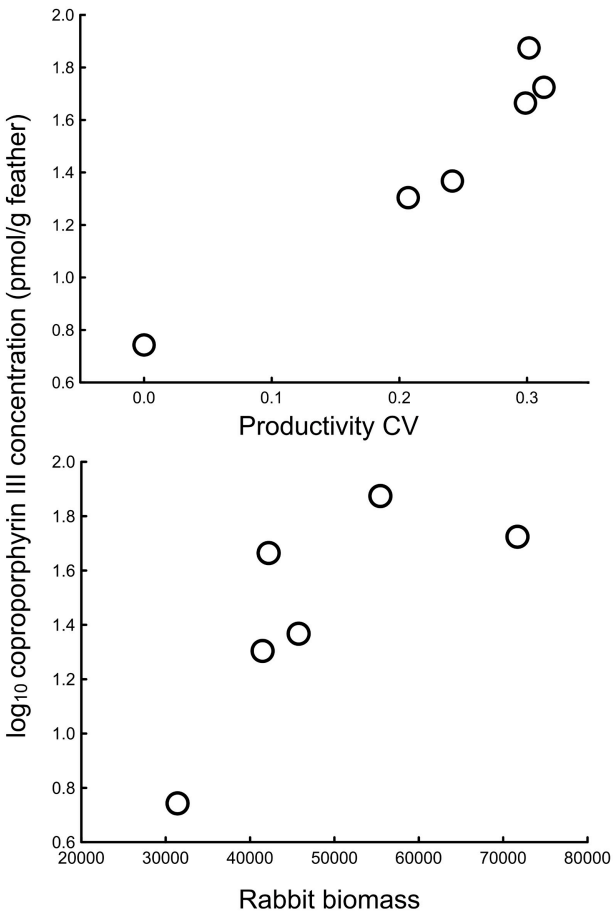


**Figure 5** Mean feather concentration of coproporphyrin III of eagle-owl fledglings per nest in relation to 2 indices of breeding site quality: Coefficient of variation of productivity (upper panel) and rabbit biomass (lower panel).

## DISCUSSION

We predicted that, if the production of feather porphyrins depends on the nutritional status of individual birds, the porphyrin content of feathers should be positively related to both fledglings' body mass and the quality of the breeding sites where they were reared. Our results support this prediction, thus confirming the signaling potential of feather porphyrins in eagle owls. Although signals whose honesty depends on trade-offs between costs and benefits of signal production (i.e. handicaps) are often condition-dependent (Cotton *et al.* 2004), the fact that the feather content of porphyrins was associated with the capacity of breeding sites to provide eagle owls with nutritional resources in addition to fledgling body condition suggests that the production of these pigments is environmentally labile and, thus, not likely to depend on the intrinsic quality of birds only, as required by handicap signals that are endogenously produced by animals. We therefore suggest that porphyrin-based feather pigmentation in eagle owls is an amplifier of the breeding site quality, so that birds reared in good breeding areas would benefit (in sexual selection terms) from the signal from their feathers rich in porphyrins to conspecifics, while birds reared in poor breeding areas would not be as successful attracting mates due to the low porphyrin contents (Hasson 1997; Galván & Sanz 2008).

These results are intriguing because we did not perceive any conspicuous porphyrin-based coloration in the feathers of eagle owls; thus, it is unclear how the signal is expressed. We contemplate 2 alternative possibilities. First, porphyrins in eagle owl feathers may produce a conspicuous coloration similar to the salmon-pink coloration produced by porphyrins in bustard feathers, which could only be observed in recently developed feathers before the pigments photodegrade because of exposure to sunlight (Galván *et al.* 2016). This possibility is supported by the fact that coproporphyrin III, which is the most abundant porphyrin in bustard feathers showing salmon-pink coloration (Galván *et al.* 2016), is also the porphyrin whose concentration is related to body mass and breeding site quality despite being less abundant than protoporpohyrin IX in eagle owl feathers. It could be speculated that the contrast against ambient light of this red (i.e. long-wavelength) reflectance of feathers may be highest at dawn, when the proportion of UV (i.e. short) wavelengths in the ambient light is higher than during midday (Håstad & Ödeen 2014). This is in line with the maximum level of vocal signaling in eagle owls, which prevalently occurs at dawn and dusk (Delgado & Penteriani 2007). It also coincides with the maximum contrast of the white throat feathers against the surrounding background (Penteriani & Delgado 2009) used by fledglings for signaling. The white throat feathers are not fully developed in eagle-owl fledglings so we could not determine if these contain porphyrins, but this is certainly a possibility. However, we did not find any perceptible color despite we inspected eagle owl fledglings when the first feathers were developing. Thus, if porphyrins produced a visually perceptible coloration, it would be so easily degradable that its function as an amplifier of territory quality would be unlikely, as it would be only functional during a very brief period of time.

A second possibility is that eagle owls have the capacity to perceive the red fluorescence of porphyrins that might naturally occur as a consequence of the exposure of feathers to the UV wavelengths of solar radiation. The retina of owls contains cone photoreceptors sensitive to long wavelengths and red oil droplets acting as cut-off filters, which suggests that these birds are capable of perceiving red colors (Bowmaker & Martin 1978). Together with their high absolute visual sensitivity (Martin 1977), these elements make it likely that eagle owls have a high capacity to perceive small increments in the red reflectance of feathers due to porphyrin natural fluorescence. Again, the duration of signal expression would be short because the fluorescence of feather porphyrins is also degraded by sunlight exposure and is only observed in recently molted feathers, but eagle owls could exhibit the signal during at least some months after molt (Weidensaul *et al.* 2011). This means that eagle owl fledglings could still signal their porphyrin-based pigmentation after leaving the nest, both during natal dispersal or when they become owners of a territory and show a high signaling activity with other (vocal) traits (Penteriani 2002; see also above). Future studies should investigate these possibilities.

Our results raise the question of why porphyrin production is mediated by health (body mass) and also by an environmental factor such as breeding site quality. Porphyrins are produced in virtually all cells as intermediates during the synthesis of heme, which serves as a prosthetic group for a number of fundamental proteins such as hemoglobin. Although this process has been mainly studied in mammals, several studies with avian cells indicate that it is very similar in birds (Goldberg *et al.* 1956; Tomio *et al.* 1970). Heme synthesis starts with the combination of the non-essential amino acid glycine with succinyl-coenzyme A to form δ-aminolevulinic acid, which suffers a series of enzy-

matic reactions that give rise to protoporphyrin IX in the mitochondria. The addition of iron to protoporphyrin IX constitutes the heme group (Ponka 1999).

Although chemically similar to protoporphyrin IX (Fig. 1), the pathway that leads to the formation of coproporphyrin III, which is the porphyrin associated with body mass and breeding site quality in eagle owls, is in part different. Coproporphyrin III is not an intermediate in the heme synthesis (Goldberg *et al.* 1956) but is neither an intermediate in its degradation pathway (Ryter & Tyrrell 2000). Coproporphyrin III is the result of the non-enzymatic oxidation of coproporphyrinogen III, an intermediate of the heme (and protoporphyrin IX) synthesis pathway, in the cytoplasm of cells (Ponka 1999; Ryter & Tyrrell 2000). Under normal circumstances, coproporphyrinogen III is oxidized by the enzyme coproporphyrinogen oxidase (EC 1.3.3.3) to form protoporphyrinogen IX and follows the conventional pathway that leads to the synthesis of heme (Ponka 1999). However, porphyrinogens are also non-enzymatically oxidized by free radicals (Woods & Calas 1989), therefore producing coproporphyrin III instead of protoporphyrinogen IX when there is a defect in the activity of coproporphyrinogen oxidase (human hereditary coproporphyria; Zaider & Bickers, 1998) or under exposure to pro-oxidant substances (Granick 1966; de Zwart *et al.* 1999).

Future studies should determine the mechanism that makes eagle owl fledglings produce coproporphyrin III in feathers. Possibilities include a deliberate induction of free radicals by fledglings in good body condition for signaling their capacity to cope with oxidative stress, and an environmental epigenetic lability in the gene encoding for coproporphyrinogen oxidase (*CPOX*; Zheng *et al.* 2014). Given the relatively small sample size and lack of knowledge on porphyrin pigmentation in birds, these possibilities need to be taken with caution, but our results provide a novel scenario for the study of animal coloration based on the levulinic metabolic route that should now be explored.

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