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Juvenile pheomelanin-based plumage colouration has evolved more frequently in carnivorous species

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Distinctive pheomelanin-based plumage colouration in juvenile birds has been proposed as a signal of immaturity to avoid aggression by older conspecifics, but recent findings suggest a detoxifying strategy. Pheomelanin synthesis implies the consumption of cysteine, a semi-essential amino acid that is necessary for the synthesis of the antioxidant glutathione (GSH) but that may be toxic if in excess in the diet. As the nestling stage probably represents a low-stress period with limited requirement for GSH protection, the synthesis of pheomelanin in developing birds may help maintain cysteine homeostasis,

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particularly in species with a high content of protein in the diet (i.e. carnivores). Here we confirm this hypothesis showing that, among 53 species of Western Palearctic birds, juvenile pheomelanin-based colouration has evolved more frequently in strictly carnivorous species than in species with other diets.

Keywords: cysteine excess, diet, juvenile plumage, pheomelanin

Bird plumage colouration usually changes with age (Booth 1990): plumage colouration becomes less dull in adult birds (Kilner 2006). However, some species do not only differ in colour intensity, but also in the pattern of pigmentation (Moreno & Soler 2011). The reasons that have favoured the evolution of these age-related changes are unclear, but understanding the mechanisms that regulate colour production may help provide insight on how pigmentation affects the way animals interact with their environment. This is because different forms of melanins, the most common pigments produced, change with age, implying different physiological constraints between juvenile and adult birds (Galván & Solano 2009).

Some comparative studies suggest that distinctive juvenile plumage has evolved as a signal of sexual immaturity to avoid aggression between conspecifics (Ligon & Hill 2013), and it has been assumed that the expression of juvenile plumage involves lower physiological costs than the expression of adult plumage (Moreno & Soler 2011). Alternatively, instead of a signalling function, the plumage displayed by juvenile birds may have evolved as a result of a detoxifying strategy (Galván 2017). This is because the reddish-chestnut colouration that is usually characteristic of the plumage of juvenile birds (del Hoyo *et al.* 1992–2002, 2003–2010) is the result of the presence of pheomelanin in feathers (Galván 2017), and the synthesis of this pigment implies the consumption of the main intracellular antioxidant and physiological reservoir of cysteine (i.e. glutathione,

GSH). Using this antioxidant to synthesize pigments, removes it from circulation which may induce chronic oxidative stress in the individual (Napolitano *et al.* 2014).

Cysteine is a semi-essential amino acid that birds largely obtain from the diet, but it causes problems such as metabolic acidosis, thinning of egg shells and poor growth when the levels in the individual are higher than needed for protein and GSH synthesis (Klasing 1998). As feathers are inert structures in which cysteine cannot exert any physiological effect, the incorporation of cysteine to feathers during the synthesis of pheomelanin may help avoid toxicity due to excess cysteine. Juvenile birds may generally suffer less physiological stress than adults (Galván *et al.* 2017) due to the reduction of physical exercise resulting from not having to find resources on their own given they are being fed by their parents (Vaanholt *et al.* 2007, 2008). These relative low stress levels may make juvenile birds more prone to the accumulation of excess cysteine, as they may have lower requirements of GSH for antioxidant protection (Galván 2017). In particular, animal food sources tend to have higher protein contents than plant-based food sources, and the latter are harder to digest (Millward 1999, Mariotti 2017). This suggests that, even at similar cysteine contents, cysteine in carnivorous diets is more physiologically available than cysteine in non-carnivorous diets. Thus, the distinctive pheomelanin-based plumage of juvenile birds may have evolved because of its potential benefits to remove excess cysteine, especially in species with a high protein content in the diet (i.e. carnivores; e.g. Bertagnolli & Wedding 1977, Nikiforova *et al.* 2002). For instance, it has recently been shown that developing Gyrfalcons *Falco rusticolus*, a strictly carnivorous bird, upregulate the expression of *CTNS*, a gene that pumps cystine (i.e. the dimer of cysteine) out of the organelles where melanin synthesis takes place in melanocytes (melanosomes), in response to a high food abundance in their rearing territories (Galván *et al.* 2017). *CTNS* expression thus avoids cysteine accumulation in melanosomes, which may in turn inhibit pheomelanin synthesis (Chiaverini *et al.* 2012). However, not all species may be able to

adjust *CTNS* expression to protein (and cysteine; Stipanuk *et al.* 2009) intake (Galván *et al.* 2017), and in the absence of *CTNS* environmental lability the synthesis of pheomelanin may constitute an alternative mechanism to help avoid cysteine accumulation and toxicity. Nevertheless, the detoxifying hypothesis for juvenile plumage has never been explored in a comparative context across species of birds. We therefore predict that juvenile plumage produced by pheomelanin-based colours and that is distinctively different from that of adults, should have evolved more frequently in carnivorous species than in non-carnivorous species. We test this prediction in 53 species of birds from the Western Palearctic that exhibit different diet habits and melanin-based plumage colour differences between adult and juvenile birds.

Methods

Data Collection

Information on plumage differences between juvenile and adult birds corresponding to 53 species from the Western Palearctic was obtained by examining colour plates in a bird identification guide (Svensson *et al.* 2000). Only birds with melanin-based plumage were selected (Galván & Wakamatsu 2016), excluding birds with plumage colouration resulting from other pigments, such as carotenoids, or feather structures because these are produced by mechanisms different from those that regulate the synthesis of the characteristic pigment of the plumage of juvenile birds, pheomelanin. We also excluded species in which diet changes between seasons, as seasonal variation may affect the protein content of the diet and plumage colouration, hence confounding the results. Birds with marked sexual dichromatism were also excluded, as sex-related effects may be difficult to control for and sexual selection could have a stronger effect on the expression of juvenile plumage than diet. Therefore, including birds with dichromatic or seasonal

plumage would prevent us from conducting comparisons in similar conditions because other evolutionary pressures may be acting. Changes in plumage colouration between juveniles and adults were scored into two categories. A score of 1 was assigned to species in which the juvenile plumage displays pheomelanin-based colouration, i.e. exhibits any of the colours that are produced by pheomelanin in feathers (orange or light brown; Galván & Wakamatsu 2016), and the adult plumage does not (Fig. 1). A score of 0 was assigned when the juvenile plumage displays any of the colours that are produced by the other main form of melanin, termed eumelanin, whose production does not consume cysteine-GSH (black, grey or dark brown; Galván & Wakamatsu 2016), and the adult plumage does not. A score of 0 was also assigned to species in which the juvenile plumage only differs from that of adults in the intensity, not in the type, of pigmentation (Fig. 1; Table S1).

Information on the diet of species was obtained from specific sources for each group of species (Cramp 1988, del Hoyo 2015, del Hoyo *et al.* 1992, 1996, 2005, Ferguson-Lees & Christie 2001, Lefranc & Worfolk 1997). Only species whose diet does not change between breeding and non-breeding seasons were considered, as variation in the protein content of diet may affect plumage colouration. Each species was categorized as strict carnivore (1) when it only consumes animal protein, a non-strict carnivore (0) when it has an omnivorous diet, or as non-carnivorous when animal protein is never present in its diet (Fig. 2; Table S1). As only one species was categorized as non-carnivorous (the European Turtle Dove *Streptopelia turtur*), we considered it as non-strict carnivorous.

Phylogenetic analyses

To test if distinctive juvenile plumage coloration produced by pheomelanin depends on the type of diet, a phylogenetic logistic regression model (Ives & Garland 2010) with the age-related plumage colour change score as a response variable and diet score as a fixed predictor was performed using the algorithm implemented by Ho and Ané (2014) in the R package *phylolm*. We obtained 1000 alternative phylogenetic trees for the 53 species of birds from www.birdtree.org (Jetz *et al.* 2012, Hackett *et al.* 2008; see Appendix S1), then constructed a consensus tree using the package *phytools* (Revell 2012) (see Fig. 2) and ultrametrized it with a value of 0.1 for the smoothing parameter δ , using the R packages *ape* (Paradis *et al.* 2004) and *phangorn* (Schliep 2011). As the response variable was binary, phylogenetic signal was measured with the parameter D developed by Fritz and Purvis (2010). D and P -values for H_0 ($D = 0$) were calculated with the package *caper* (Orme *et al.* 2013): $D = 1$ is indicative of a random mode of evolution (i.e. no phylogenetic signal), whereas $D = 0$ is indicative of a Brownian motion mode of evolution, in which changes in the trait along the phylogeny are proportional to the degree of relatedness among species. D can also be < 0 in highly clustered traits and > 1 when phylogenetic overdispersion exists (Fritz & Purvis 2010).

Results

Pheomelanin-based plumage pigmentation during the juvenile stage was observed in 29 of 39 (74%) strict carnivorous species and only 2 of 14 (14%) non-strict carnivorous species (Fig. 3). Accordingly, the phylogenetic logistic regression model recovered a significant ($b = 2.32$, $P = 0.003$; Fig. 3) relationship between diet and juvenile plumage colouration. This result indicates that the presence of juvenile plumage colouration produced by pheomelanin is higher among strict carnivorous than among non-carnivorous

birds. The phylogenetic signal for plumage colour score ($D = -0.40$) was not statistically different from zero ($P = 0.746$), suggesting that the presence of pheomelanin-based plumage colouration in the juvenile stage of birds is a conserved trait.

Discussion

When the plumage of juvenile birds is distinctively different from that of adults, it commonly exhibits orange and light brown colours, as can be inferred by an examination of bird illustrations (Svensson *et al.* 2000). These colours are produced by the presence of pheomelanin in feathers (Galván & Wakamatsu 2016). The type of juvenile plumage colouration (i.e. more or less presence of pheomelanin in juveniles than in adults) exhibits phylogenetic conservatism, suggesting that juvenile plumage may provide similar functional benefits to all species and thus selection pressures are constant along the phylogeny. This is because, when some but not all species in a phylogeny achieve a particular character-state in relation to a trait, this results in a loss of phylogenetic signal (i.e. the signal is reduced relative to that expected from a Brownian motion model of evolution; Blomberg *et al.* 2003).

Our study also suggests that pheomelanin-based plumage in juvenile birds has evolved more frequently in strict carnivorous species than in species that include food sources other than animal proteins in their diet. We propose that this is due to the potential adaptive benefit that the synthesis of pheomelanin for pigmentation could represent, as it may help avoid the toxicity of excess cysteine with high protein content in the diet. The production of pheomelanin may thus contribute to cysteine homeostasis when the expression of the gene *CTNS* cannot be adjusted to protein intake, as *CTNS* lability is not expected to have evolved in every species (Galván *et al.* 2017). This potential benefit may be particularly advantageous for birds developing feathers during the nestling stage, a

period of low physical activity in which they are fed by their parents. Exercise can increase oxidative stress (Vaanholt *et al.* 2007, 2008), thus the absence of parental and feeding effort results in the nestling stage constituting a period of relatively low stress as compared to post-fledging stages when juveniles must find food on their own with a high expenditure of energetic resources (Weimerskirch *et al.* 2003). This may explain the abundance of pheomelanin-based plumage in juvenile birds, which probably have a relative low requirement of antioxidant resources. Recent findings in birds suggest a positive association between plasma protein concentration and the expression of pheomelanin-based plumage colouration (Minias *et al.* 2018). As total plasma protein levels increases with the intake of dietary proteins (Leveille & Sauberlich 1961), this result could be in agreement with the detoxifying hypothesis of pheomelanin-based pigmentation as articulate here.

While our results suggest that pheomelanin-based pigmentation has evolved more frequently in strictly carnivorous species, variability still exists across species and, accordingly, the phylogenetic signal for the type of juvenile plumage colouration was significant (i.e. D is not statistically different from zero) but not as strong as it could be if the estimated value for D was near 0. Thus, there are closely related species that have a strict carnivorous diet but differ in juvenile plumage pigmentation.

An example of this variability is represented by the Spanish Imperial Eagle *Aquila adalberti* and the Golden Eagle *Aquila chrysaetos*, two congeneric raptor species that differ in juvenile pigmentation. The first plumage of the Spanish Imperial Eagle is full orange, a colour conferred by the presence of pheomelanin in feathers (Galván & Wakamatsu 2016), and it changes to a fully black plumage, a colour conferred by eumelanin, in adulthood (Fig. 1a). The Golden Eagle presents a reverse pattern, as the first plumage is almost fully black, it subsequently changes to a lighter brown-orange plumage in adulthood (Fig. 1b). It could be thus said that juvenile Spanish Eagles are

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pheomelanic, whereas juvenile golden eagles are eumelanic. As all raptors, both species are strictly carnivorous. We suggest that the different pigmentation patterns may be related to the different trophic ecology of the species. The Spanish Imperial Eagle is a specialist that preys preferentially on rabbits (Ferrer & Negro 2004), whereas Golden Eagles prey on a much wider diversity of species (Watson 2010). The predictability of food resources is higher for specialists than for generalists (Overington *et al.* 2008), and increases with food abundance in raptors (Millon *et al.* 2008). Pheomelanin synthesis may help avoid excess dietary cysteine but, if cysteine is not in excess, it may cause oxidative stress by consuming GSH (Napolitano *et al.* 2014). If food availability and predictability are higher for Spanish Imperial Eagles than for Golden Eagles, it is then likely that natural selection has favoured the evolution of pheomelanin-based pigmentation in the former but not in the latter. Indeed, the sister species of the Spanish Imperial Eagle is the Eastern Imperial Eagle *Aquila heliaca*, which consumes a more diverse diet (Katzner *et al.* 2005) and the juvenile plumage is less intensely orange (Fig. 1c). A non-excluding alternative hypothesis is that these species differ in the permeability of *CTNS* expression to food abundance (Galván *et al.* 2017). Future studies should test these possibilities.

Understanding the physiological mechanisms of colour production helps to explain the evolution of plumage diversity (Delhey 2015). This study seeks to provide an explanation for differences between the plumage pigmentation of juvenile and adult birds as a physiological response to diet. Although we focused our study on birds, melanins are the most abundant pigments in animals (McGraw 2006), thus these findings will contribute to our understanding of the evolution of pigmentation phenotypes in other taxa.

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Figure legends

Figure 1. Images of two congeneric species of strict carnivorous birds with different age-related plumage pigmentation changes. In the print version Black colours represent eumelanin-based plumage colouration while grey colours correspond to pheomelanin-based colouration. (a): juvenile (left) and adult (right) Spanish Imperial Eagles *Aquila adalberti*. Figure taken from Negro *et al.* 2018 (CC BY 4.0 license: <https://creativecommons.org/licenses/by/4.0/>). (b): juvenile (above; photograph courtesy of Carlos Pacheco) and adult (below; photograph by David Illig: <https://flic.kr/p/9usE42>; license CC BY-NC 2.0: <https://creativecommons.org/licenses/by-nc/2.0/>) Golden Eagles *Aquila chrysaetos*. (c): juvenile (left) and adult (right) Eastern Imperial Eagles *Aquila heliaca*. (right; photograph by Andi Li: https://www.flickr.com/photos/andy_li/; CC BY-NC 2.0 license: <https://creativecommons.org/licenses/by-nd/2.0/>).

Figure 2. Consensus phylogenetic tree for the 53 species of birds included in the study obtained from www.birdtree.org. Branch lengths are proportional to nucleotide substitutions. Juvenile colour score values (black: non-pheomelanin plumage coloration; white: pheomelanin-based plumage coloration) and diet habitat (lines: non-strict

carnivorous species; circles: carnivorous species) for the 53 species of birds included in the study.

Figure 3. Proportion of species exhibiting pheomelanic-based juvenile plumage colouration (black) relative to the proportion of species in which juveniles exhibit other plumage colouration (grey) for strict carnivorous ($n = 39$) and non-strict carnivorous species ($n = 14$).