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Dehydrolutein: a metabolically derived carotenoid never observed in raptors

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Carotenoids are fat-soluble pigments synthesised by photosynthetic organisms (Brush, 1990). Conversely, animals are incapable of synthesizing carotenoids *de novo*, and they must obtain them through their diet. However, some animal species are able to make some alterations to the basic chemical structure, converting ingested carotenoids into more oxidized and differently coloured forms (Schiedt, 1998).

Over 600 types of carotenoids have been classified so far and many diverse health modulating properties have been ascribed to these compounds in animals (e. g., Olson and Owens, 1998; Møller et al., 2000; but see Costantini and Møller, 2008). In birds, carotenoids are also often used in sexual visual displays through deposition in skin or feathers, where they are responsible for most of the yellow to red colourations that we observe (Olson and Owens, 1998; Tella et al., 2004; Hill and McGraw, 2006a, b).

To understand the physiological mechanisms underlying carotenoid-based colourations or the physiological functions of carotenoids, it is pivotal to document the carotenoid profile, i. e., carotenoids accumulated by a species. However, most studies conducted thus far analysed in detail carotenoids in passerine birds (e. g., Stradi et al., 1996; Hill and McGraw, 2004), while less attention has been directed to other avian groups, such as raptorial birds. As far as we know, the carotenoid profile of only three diurnal raptor species have been described in detail (*Falco sparverius* in Bortolotti et al., 1996; *Neophron percnopterus* in Negro et al., 2002; *Falco tinnunculus* in Casagrande et al., 2006), while only total plasma carotenoid concentration has been measured in other raptor species (Tella et al., 2004). It was found that birds of prey accumulate untransformed carotenoids (lutein and zeaxanthin) in the bare parts (legs, bill) but not in feathers. Furthermore, it was found that skin colouration correlates with quality attributes of male Eurasian kestrels, such as hunting skills and habitat quality, and with body size and territorial quality of male American kestrels (Bostrom and Ritchinson, 2006; Casagrande et al., 2006). Overall, these studies hypothesised that (i) raptors could be unable to metabolically modify ingested carotenoids and (ii) carotenoid-based skin colourations could be a honest signal of the male's quality in some raptor species.

Given the paucity of studies on carotenoids in raptors, this study sought to describe the carotenoid profile of tissues (feathers, tarsi skin, and/or blood) of five common European raptor species. More specifically, we have described the carotenoid profile of the two diurnal raptor species, the sparrowhawk *Accipiter nisus* (Accipitriformes) and the peregrine falcon *Falco peregrines* (Falconiformes), and of the three nocturnal raptor species (Strigiformes), the little owl *Athene noctua* (Strigidae), the tawny owl *Strix aluco* (Strigidae), and the barn owl *Tyto alba* (Tytonidae). We chose to analyse the carotenoid profile of feathers and skin because carotenoid-based colourations of both these tissues are likely to be relevant in mate choice of birds (Olson and Owens, 2005; Casagrande et al., 2006). Finally, we chose to study these species because (i) these are common European raptor species, which were object of many ecological but not physiological studies, (ii) diurnal but not nocturnal raptors show skin colourations, whose biochemical nature is, however, unknown, (iii) to cover a wide taxonomic range into the birds of prey group.

1 Materials and methods

1.1 Biological materials

Fresh tarsus skins and breast feathers were collected

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in March 2006 from two sparrowhawks *A. nisus* (one male and one female), one peregrine falcon *F. peregrinus* (female), and two barn owls *T. alba* (one male and one female) in carcasses sent to CEH Monks Wood by members of the public in Britain. The age of these birds was not known. Data on circulating carotenoids in sparrowhawks and barn owls were taken from literature (Tella et al., 2004); therefore, we did not collect any blood samples from these two species. No serum sample for the peregrine falcon was available. Breast feathers and blood samples were collected in September 2007 from two adult little owls *A. noctua* (one male and one female), which were obtained from local rehabilitation centers. Both little owls had stabilized bone fractures in the wings and could not have been released into the wild. However, they were in good condition. Both birds were fed with one-day-old cockerels and were maintained in the facilities for around one year before blood collection. Finally, the carotenoid profile was analysed in breast feathers and blood collected in September 2007 from one juvenile tawny owl *S. aluco* (male). No skin samples for little owls and tawny owls were available. Tissue samples collected from captivity or dead birds were already proved to be reliable to determine the carotenoid profile in birds of prey (e. g., Negro et al., 2002; Casagrande et al., 2006).

1.2 Chemicals and reagents

Methanol, acetonitrile and acetone were obtained from Merck (Darmstadt, Germany) and the solvents were filtered through a Millipore (Milford, MA, USA) membrane filter (13 mm diameter, 0.5 μm porosity).

1.3 Extraction procedure

The feather carotenoids were extracted and concentrated as follows: we carefully washed ca. 5 mg of coloured barbules with hexane on a glass filter and finely ground the barbules in a ZrO container in the presence of 2 mL methanol, 25 spheres with a micronizer Retsch MM301 (Hann, Germany) for 10 minutes at room temperature at 15 cps. The solid residues (inorganic salts and feather proteins) were completely removed from the solution containing carotenoids through filtration on Sep-Pak C18 cartridges (Waters Millipore, Milford, MA). The filtrate containing the carotenoid pigments was evaporated under reduced pressure at room temperature, and then dissolved the ensuing residue in 200 μL of acetone. After freezing for 3 h at −78℃, we filtered the supernatant. The clear carotenoid solution was evaporated under a stream of dry nitrogen and the residue dissolved in the HPLC mobile phase. For the isolation of carotenoids from skins, the same protocol developed for feathers was used except that ca. 50 mg of skin was used.

Serum carotenoids were extracted and concentrated as follows: 50 μL of serum were dissolved in 200 μL of acetone. Then the solution was firstly sonicated for 30 seconds and secondly centrifuged at 14000 r/min for one minute. After this, the supernatant was taken and evaporated under a stream of dry nitrogen. The residue was then dissolved in the HPLC mobile phase.

1.4 HPLC-uv/vis

A Waters 600E instrument equipped with a quaternary pump was used (MA, USA). Carotenoid analyses were carried out by using Simmetry Shield RP-18 columns (5 μm) (250 mm × 4.6 mm I. D.) maintained at 30℃ by a column block heater (model 7970, Hichrom Ltd., Reading, UK). Samples were injected with a Rheodyne 7125 valve equipped with 20 μL loop (Rheodyne, Rohnent Park, CA, USA. We used a methanol/acetonitrile (5/95, v/v) mobile phase running at 1 Ml/min to characterise the carotenoids present in samples. Data were acquired for the wavelength range 230 to 600 nm, using a photodiode-array detector (waters 2996), and integrated areas under the peaks detected at 450 nm. Three-dimensional chromatograms were recorded using Empower 2 software (Waters). All carotenoids were identified by UV-Vis lambda max value, molecular weight and co-elution with certified carotenoids (Carotenature, Lupsingen, Switzerland).

1.5 HPLC/MS

A Thermo Finnigan instrument LCQ Advantage Series (Thermo Finnigan, San Jose, CA, USA), equipped with a quaternary pump (Surveyor), a diode-array detector, an electrospray ionisation source (ESI), and an ion trap analyzer (positive mode) was used for LC-MS analyses and to obtain UV-vis and mass spectra of eluted carotenoids. Carotenoid separations were carried out (HPLC analyses) using Simmetry Shield RP-18 columns RP-18 columns (5 μm) (250 mm × 4 mm I. D.) maintained at 30℃ by a column block heater. A methanol/acetonitrile (5/95, v/v) mobile phase running at 1 mL/min was used to characterise the carotenoids present in samples. The samples were injected using a Rheodyne (model 7125) valve equipped with a 20 μL loop. Spectral data for the wavelength range 230 to 600 nm were acquired using a photodiode-array detector (Surveyor), and integrated areas under the peaks detected at 450 nm. Three-dimensional chromatograms were recorded using Thermo Xcalibur software.

2 Results

The analysis of the tarsus skin revealed the presence of the three xanthophylls lutein (male and female: 166.73 and 132 μg/g), zeaxanthin (male and female: 19.61 and 3.63 μg/g), and 3′-dehydrolutein (male and female: 11.80 and 55.22 μg/g) in both sparrowhawks, giving values of total carotenoids of 198.14 and 190.85 μg/g, respectively. The same carotenoid profile was found in the female peregrine falcon: lutein, 115 μg/g; zeaxanthin, 10.04 μg/g; 3′-dehydrolutein, 110.07 μg/g (total carotenoids: 235.11 μg/g). Levels of carotenoids present in the tarsus skin of barn owls were not enough for

a correct identification. Serum sample of the tawny owl contained lutein (0.16 μg/mL) and zeaxanthin (0.03 μg/mL), while the concentration of 3′-dehydrolutein (0.01 μg/mL) was not enough for a correct identification (total carotenoids: 0.20 μg/mL). Finally, breast feathers of all the species considered did not contain any traces of carotenoids. Serum samples of the little owls contained lutein (male and female: 4.63 and 1.48 μg/mL), zeaxanthin (male and female: 1.08 and 0.38 μg/mL), and 3′-dehydrolutein (male and female: 0.14 and 0.07 μg/mL), giving values of total carotenoids of 5.85 and 1.93 μg/mL.

Our data agree with the structure of 3′-dehydrolutein VIS λ 447, 475 nm. MS m/z 567 ([M-H]$^+$, C40H54O2), lutein VIS λ 422, 446, 475 nm. MS m/z 569 ([M-H]$^+$, C40H56O2) and zeaxanthin VIS λ 454, 480 nm. MS m/z 569 ([M-H]$^+$, C40H56O2) (Fig.1). All carotenoids identified did not separate from the standard carotenoids used under our chromatographic conditions.

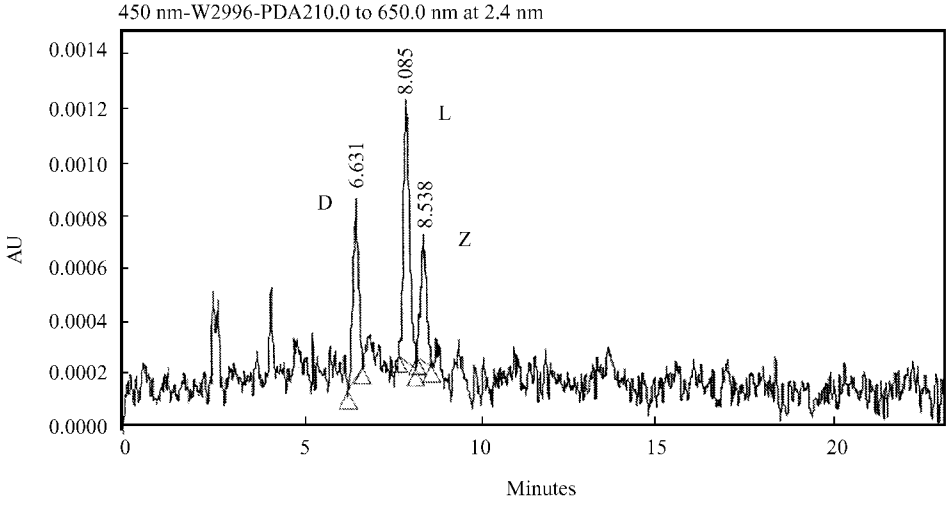

Fig.1 PDA Chromatogram of an extract at 450 nm

The extract was got mixing the extracts from sparrowhawks, peregrine falcon and barn owls. D = Dehydrolutein, L = Lutein, Z = Zeaxanthin.

3 Discussion

The main result of our study is that some birds of prey accumulate 3′-dehydrolutein, showing that raptorial birds can metabolically modify dietary carotenoids. In fact, dehydrolutein is a metabolically derived carotenoid that has not been found before in studies on raptor species. More specifically, dehydrolutein is a yellow carotenoid derived from enzymatic dehydrogenation of dietary carotenoids. Dehydrolutein was identified in feathers of some bird species (e. g., Stradi et al., 1996; Massa and Stradi, 1999; McGraw and Schuetz, 2004; Andersson et al., 2006). In chickens *Gallus gallus domesticus*, it has been found that dehydrolutein physiologically derives from zeaxanthin (Schiedt, 1998). While this molecule has been detected in yellow lipochromes of passerine birds, it is not essential for all the yellow colourations. For example, dehydrolutein has been found in the robin *Erythacus rubecula*, where there is no yellow external colouration (Massa and Stradi, 1999). In the present study, dehydrolutein was also found in nocturnal raptors, which lack carotenoid-based colourations. Given these points, the role of dehydrolutein in animal colouration and communication is far from clear and deserves further studies.

While we cannot exclude that some dehydrolutein may be absorbed from prey (i. e., carotenoids modified already in the prey), we are confident that our results actually show that some raptors are capable of metabolically modifying carotenoids. Dehydrolutein has been found rarely in birds and, more importantly for our study, it has never been found in many animal species (rodents and lizards) raptors feed on (see Czeczuga, 1980; Czeczuga and Malzahn, 1980; Massa and Stradi, 1999). One more point that supports our conclusions is that kestrels have a larger food-niche breadth than that of the peregrine falcon and of the sparrowhawk, but their food habits partly overlap (e. g., passerine birds). Similarly, the diet of kestrels partly overlaps that of the tawny owl (e. g., Marti et al., 1993). Hence, assuming that dehydrolutein is ingested, one would expect kestrels to accumulate dehydrolutein rather than the peregrine falcon, the sparrowhawk, or the tawny owl. Actually, the contrary is what we found, so indirectly supporting the evidence that some raptor species are capable of metabolically deriving dehydrolutein.

A comparison of circulating carotenoids between the present species and those taken from the literature is not very meaningful given the heterogeneity of the overall sample and the number of factors that may not be

controlled for. Not only food quality may differ between wild and captive birds, but also differences in the sampling period (e. g., spring vs. winter) may make comparison of carotenoid concentration difficult. For example, Tella et al. (2004) sampled all the birds during winter, when it is predicted that circulating carotenoids are at minimum level and they are not allocated to sexual colourations. Conversely, Casagrande et al. (2006) sampled birds during the reproductive season. While this heterogeneity between studies, some considerations may still be drawn. Differences in levels of circulating carotenoids between diurnal and nocturnal raptor species seem to be poorly explained by diet differences. Barn owls and tawny owls mainly feed on rodents, which are a poor source of carotenoids (Czeczuga and Malzahn, 1980; Bortolotti et al., 2000; Casagrande et al., 2006). However, circulating carotenoids previously found in one kestrel population feeding on rodents (median concentration of 34.78 μg/g in males and of 32.51 μg/g in females; Casagrande et al., 2006) were much higher than those found in both owl species (barn owl in Tella et al., 2004; tawny owl in the present study). Similarly, little owls maintained in captivity on a diet of one-day-cockerels showed lower mean levels of circulating carotenoids (mean value of 3.89 μg/mL) than American or Eurasian kestrels kept on a similar diet (Negro et al., 1998; 40.72 μg/mL in Costantini et al., 2007). One comparative study (Tella et al., 2004) suggested that some of the interspecific differences in plasma carotenoid concentrations may be explained by differences in the selective absorption processes in the small intestine (e. g., appropriate lipoproteins that transport carotenoids from the gut to the blood), which are known to vary from species to species (Trams, 1969; Yang and Tume, 1993; Olson and Owens, 1998). We do not know whether diurnal and nocturnal raptors differ in the selective absorption processes in the small intestine. However, we highlight that studies on this topic may be warranted of attention.

From a phylogenetic point of view, McGraw and Schuetz (2004) thoroughly examined the evolutionary trajectory of carotenoid metabolism in estrildid finches. The authors found species incapable of transforming dietary carotenoids only in the most derived clade, suggesting that the loss of ability to metabolize dietary carotenoids can be labile and likely shaped by the relative costs and benefits of colour signalling across different species (McGraw and Schuetz, 2004). Another study on Passeriformes and Galliformes showed experimentally that species of the more recently derived order (Passeriformes) more efficiently extract and accumulate carotenoids from food than do species of the more ancient lineage (Galliformes) (McGraw, 2005). Similarly, Tella et al. (2004) proposed the existence of physiological constraints to the acquisition of carotenoids, which are linked to the phylogeny of the species.

Our study suggests that the ability of raptors to metabolically modify dietary carotenoids seems to be phylogenetically labile. In fact, while the species belonging to the genera *Accipiter* and *Falco* were documented to be phylogenetically unrelated (Griffiths, 1997, 1999; Griffiths et al., 2004), *Falco peregrinus* shows a carotenoid profile the same as that of *Accipiter nisus* (both have L, Z and D), but different from those of its phylogenetically related species *Falco sparverius* and *Falco tinnunculus* (both have L and Z only; Bortolotti et al., 1996; Casagrande et al., 2006). *Accipiter nisus* and *Falco peregrinus* also show a carotenoid profile the same as those of the two Strigiformes species, *Strix aluco* and *Athene noctua*, but different from that of the more phylogenetically related species, *Neophron percnopterus* (Griffiths, 1994; Negro et al., 2002). These data suggest that the capability of birds of prey to metabolically transform carotenoids could have arisen by convergent evolution. However, our small sample size does not allow us to be conclusive, hence we encourage future comparative and experimental studies to test this hypothesis. In conclusion, we have shown that (i) some raptor species can metabolically modify dietary carotenoids, (ii) phylogenetically related species may have different carotenoid profiles.

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