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# Reptiles Produce Pheomelanin: Evidence in the Eastern Hermann's Tortoise (*Eurotestudo boettgeri*)

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Abstract.—**Reptiles, supposedly, do not produce pheomelanin pigments. Because this claim is based on rather weak evidence, we measured the shell pheomelanin content in the Hermann's Tortoise (*Eurotestudo boettgeri*). In contrast to expectation, we detected a substantial amount of this pigment. Given the recent interest in the adaptive function of melanin-based color traits, our study opens new avenues of research in reptiles.**

In vertebrates, variation in coloration between species and individuals belonging to the same species is mainly attributable to differential deposition of reddish-brown pheomelanin and to black/grey eumelanin pigments (Majerus, 1998). Knowledge of which pigment is responsible for a given color patch is key to understanding the adaptive function of color variation. In vertebrates, melanin-based coloration is associated with a number of ecologically relevant traits, and sign and magnitude of natural and sexual selection exerted on melanin-based coloration can be species or trait specific (Jawor and Breitwisch, 2003; Roulin, 2004; Meunier et al., 2011). Melanin-based coloration can play a major role in predator–prey interactions with eumelanin- and pheomelanin-based coloration conferring camouflage in different habitats (Hoekstra et al., 2005). The physical and biological properties of melanin could also be pigment specific, because melanin is known to protect the external body surface against abrasion (Bonser, 1995), solar radiation (Clusella Trullas et al., 2007), and pathogens (Mackintosh, 2001). In some species, melanin-based coloration has been shown to signal aspects of quality and is, therefore, used as mate choice criterion (Pryke and Griffith, 2007). Although in most of the investigated animal species darker eumelanic individuals were found to be more aggressive and sexually active than paler conspecifics, the adaptive function of pheomelanin-based coloration is still poorly understood (Ducrest et al., 2008).

The genetics of melanogenesis are very well known (Ito and Wakamatsu, 2011), which stimulated researchers to link phenotype to genotype in wild animals (Mundy et al., 2004; Rosenblum et al., 2004; Hoekstra, 2006). Melanogenesis involves complex machinery. By binding to the melanocortin receptor 1, melanocortins induce the production of eumelanin but block the production of pheomelanin and the opposite with agouti signaling protein (ASIP) (Lin and Fisher, 2007; Le Pape et al., 2009). Interestingly, melanocortins have pleiotropic effects on a large number of physiological and behavioral functions (Ducrest et al., 2008). Thus, eumelanin- and pheomelanin-based coloration may be associated with the same phenotypic traits but in opposite directions (Roulin et al., 2011).

There are few reports regarding melanin determination in vertebrates except for mammals and birds (Prota, 1992; Ito, 1998; Ito and Wakamatsu, 2003, 2006). High levels of pheomelanin are found only in yellow to red hairs of mammals and in reddish feathers of birds. Detectable levels of pheomelanin are also detected in human skin regardless of race, color, and skin type. However, eumelanin is always the major constituent of epidermal melanin. In different species of amphibians and reptiles, it has been demonstrated that the pigmented macrophages of the liver are able to synthesize melanins (Cicero et al., 1982, 1989; Scalia et al., 1988). These authors identified pyrrole-2,3,5-tricarboxylic acid (PTCA) and pyrrole-2,3-dicarboxylic acid (PDCA), degradation products of eumelanin, and classified the liver melanin as indole melanin. Gallone et al. (2007) described, in amphibians (*Rana esculenta*), that liver melanin is composed of 5,6-dihydroxyindole (DHI)-rich eumelanin showing characteristics very similar to those of the sepia melanin, although it contained little pheomelanin (Table 1). Recently, Wolnicka-Glubisz et al. (2012) used EPR spectra and reported that pheomelanin is present in the dorsal skin of adult frogs (*Hymenochirus boettgeri*). However, they did not quantify the melanin contents. Adachi et al. (2005) reported that the 4-amino-3-hydroxyphenylalanine (4-AHP) level of Red Seabream (*Pagrus major*) in skin was found to be below the detection limit of 0.015 ng/mg dry skin, regardless of the season and sex. On the other hand, the average level of PTCA was 4.7 ng/mg dry weight. They demonstrated that pheomelanin has not been identified in any fish.

In reptiles, coloration is often structural or attributable to carotenoid and pteridine pigments (Olsson et al., 2007). Reptiles are thought to produce only eumelanin but not pheomelanin. Many contemporary studies (e.g., Grether et al., 2004; Rosenblum et al., 2004) cite older publications (e.g., Fujii, 1993; Ito and Wakamatsu, 2003) to suggest that reptiles do not produce pheomelanin although these studies did not provide direct evidence. Given the interest in melanin-based color traits among evolutionary ecologists, it is important to either confirm or reject the hypothesis that reptiles have no pheomelanin. To address this question, we performed a study on the Eastern Hermann's tortoise (*Eurotestudo boettgeri)*. This species is native to the Balkans and Greece and displays a high variation in melanin-based coloration between and within populations, as well as within a clutch (Willemsen and Hailey, 1999; Vetter, 2006). The dark shell pigmentation, probably caused by eumelanin, usually covers less than 50% of each scute, and the rest, which is yellowish to brownish, may be attributable to pheomelanin (Fig. 1).

## Materials and Methods

Our aim was to demonstrate the presence or absence of pheomelanin pigments in the shell of Hermann's Tortoise. For this observational study, we sampled thin shavings from the surface of black, brown, and yellow parts of the shell of three Hermann's Tortoises (adult male, adult female, and juvenile). These tortoises belonged to one of the authors (A. Mafli; for

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TABLE 1. Contents of melanin markers in liver melanin of amphibian, red seabream, and sepia melanin. PTCA and PDCA are degradation markers by acid $KMnO_4$ axidation and/or alkaline $H_2O_2$ oxidation, respectively. 4-AHP is a degradation marker by HI hydrolysis.

| Sample | PTCA by acidic $KMnO_4$ oxidation (ng/mg) | 4-AHP by HI hydrolysis (ng/mg) | PTCA by Alkaline $H_2O_2$ Oxidation (ng/mg) | PDCA by Alkaline $H_2O_2$ Oxidation (ng/mg) | PTCA/ PDCA (ng/mg) |
|---|---|---|---|---|---|
| Liver melanin of *R. esculenta* L.[a] | 530 | 42 | 1210 | 27 | 45 |
| Red Seabream[b] | 4.7 | 0.015 | | | |
| Sepia melanin[a] | 530 | <10 | 11300 | 190 | 59 |

[a] Gallone et al., 2007.
[b] Adachi et al., 2005.

further details, also see Mafli et al., 2011) (Fig. 1). Eumelanin content was analyzed by HPLC-chromatography as the permanganate oxidation product PTCA and pheomelanin content was given as the hydriodic acid hydrolysis product 4-AHP (Wakamatsu et al., 2002; Ito and Wakamatsu, 2003).

The thin shaving samples, immersed in 1 ml milli-Q water were homogenized with a glass homogenizer. Permanganate oxidation was performed in duplicate at room temperature in 800 μl of 1 mol/L $H_2SO_4$; 100 μl of liver homogenate (50 mg/ml) was included in the oxidation medium; and 10 μl of 3% $KMnO_4$ was added at regular interval as in the original method (Ito and Fujita, 1985; Ito and Wakamatsu, 1994). $Na_2SO_3$ (100 μl) was added, and the reaction mixture was extracted twice using 7 ml of ether. Residue, after evaporating ether extracts, was taken up in 200 μl of milli-Q water and centrifuged. An aliquot of 80 μl was injected into the HPLC system. Results are averages for duplicate analyses. The HPLC system consisted of a JASCO 880-PU liquid chromatograph (JASCO Co., Tokyo, Japan), a Shiseido C18 column (Capcell Pak Type MG; 4.6 × 250 mm; 5 μm particle size; Shiseido, Tokyo, Japan), and a JASCO UV detector monitored at 269 nm. The mobile phase was 0.1 mol/L potassium phosphate buffer (pH 2.1) : methanol, 99 : 1 (v/v). Analyses were performed at 45°C at a flow rate of 0.7 ml/min. Permanganate oxidation product, PTCA (pyrrole-2,3-5-tricarboxylic acid) was measured for eumelanin content.

Hydriodic acid reduction was performed by heating a mixture of 100 μl of a sample homogenate, 30 μl of 30% $H_3PO_2$, and 500 μl of 57% HI in a screw-capped tube at 130°C for 20 h, after which the mixture was cooled. A 100 μl portion of the hydrolysate was transferred to a test tube and evaporated to dryness using a vacuum pump connected to a dry ice-cooled vacuum trap and two filter flasks containing NaOH pellets. The residue was dissolved in 200 μl of 0.1 mol/L HCl; 10 μl of the solution was analyzed on the HPLC system as described below. The HPLC system consisting of a JASCO 880-PU liquid chromatograph, a JASCO C18 column (JASCO Catechol pak; 4.6 × 150 mm; 7 μm particle size; JASCO, Tokyo, Japan) with AHP buffer : methanol, (98 : 2 [v/v]) at 35°C, with an electrochemical detector (ECD-300, EICOM, Kyoto, Japan) set at +500 mV versus Ag/AgCl electrode, at a flow rate of 0.7 ml/min. The AHP buffer consisted of 0.1 mol/L sodium citrate buffer, pH 3.0, containing 1 mmol/L sodium octanesulfonate and 2% EDTA.2Na. Finally, HI hydrolysis product, 4-AHP was measured for pheomelanin content (Wakamatsu et al., 2002; Ito and Wakamatsu, 2003).

FIG. 1. Eastern Hermann's Tortoise (*Eurotestudo boettgeri*) displaying yellow with black parts. A distal scute at the center of the animal is partly brown. Photograph by A. Mafli.

## RESULTS

The eumelanin/pheomelanin ratio increased along with the change from yellowish to black coloration of the shell (Table 2).

TABLE 2. Content of melanin markers of the yellow, brown, and black parts of the shell in three Hermann's tortoises. Eumelanin content was given by the permanganate oxidation product PTCA and pheomelanin content by the hydriodic acid hydrolysis product 4-AHP.

| | Eumelanin PTCA (ng/mg) | Pheomelanin 4-AHP (ng/mg) | PTCA/4-AHP ratio |
|---|---|---|---|
| Adult male | | | |
| Yellow part | <0.14[a] | 9.72 | <0.014 |
| Brown part | 28.8 | 9.46 | 3.04 |
| Black part | 40 | 5.66 | 7.07 |
| Adult female | | | |
| Yellow part | <0.14[a] | 22.2 | <0.006 |
| Brown part | 65.4 | 8.56 | 7.64 |
| Black part | 52.6 | 3.87 | 13.59 |
| Juvenile | | | |
| Yellow part | <0.14[a] | 9.45 | <0.014 |
| Brown part | 96.9 | 11.8 | 8.21 |
| Black part | 249 | 7.45 | 33.42 |

[a] Values below the detection limit of HPLC determination.

The yellow part of the shell contained mainly pheomelanin pigments. Thus, contrary to the claim that reptiles do not produce pheomelanin, we detected a substantial amount of this pigment in the scutes of Eastern Hermann's Tortoises.

## Discussion

The finding that reptiles do produce pheomelanin pigments is important for several reasons. First, in reptiles yellow/reddish coloration can be attributable to the deposition of carotenoids, pterins, and pheomelanin (Macedonia et al., 2000). Our paper demonstrates that, without analyzing pigments, we cannot discount pheomelanin and assume that the yellow/reddish coloration in reptiles is carotenoid or pterin based. This is important because the carotenoid pigments are derived from the diet, whereas pheomelanin pigments are endogeneously produced.

Second, mammals and birds are already known to produce pheomelanin. Given the close phylogenetic position of birds and reptiles, absence of pheomelanin in reptiles would have implied a secondary loss in the production of this pigment. Our demonstration of the presence of pheomelanin highlights the need for additional data to be collected across a phylogenetically diverse groups of reptiles.

Third, the adaptive function of pheomelanin-based coloration is less well known than of eumelanin-based coloration (Galvan and Solano, 2009; Roulin, 2009). Because reptiles are used frequently by researchers to study the role of natural and sexual selection in the evolution of color traits (e.g., Olsson et al., 2007; Lepetz et al., 2009), we may soon obtain more information on the signaling value of pheomelanin-based coloration in this group of animals. Finally, melanin-based color traits are frequently associated with behavioral traits (Ducrest et al., 2008), but so far most evidence comes from the study of black eumelanic traits. Given that reptiles often vary in both black and yellow/reddish coloration, they appear to be appropriate model organisms to investigate further the relative role of eumelanin and pheomelanin in generating covariation between color and behavioral, morphological, physiological, and life-history traits.

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