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On the Cover: This male black-eared wheatear (*Oenanthe hispanica*) was photographed at Perabad Mountains in Ciudad Real, Spain, by Rafael Palomo Santana. The bird's plumage coloration is generated by different chemical forms of melanins.

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BRIEF COMMUNICATION

Melanin Chemistry and the Ecology of Stress

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ABSTRACT

Knowledge of melanin chemistry has important implications for the study of the evolutionary ecology of animal pigmentation, but the actual chemical diversity of these widely expressed biological pigments has been largely overlooked. Considering all melanin forms and the different conditions of endogenous oxidative stress during their synthesis provides information about physiological costs and benefits of different pigmentation patterns and opens a new perspective to understanding the evolution of color phenotypes in animals.

Introduction

Melanins are the most widely expressed biological pigments in nature, found in virtually all organisms. The presence of melanins in both the integument and internal structures is essential for the development of life on earth, as melanization constitutes the main physiological response of animals and microorganisms against the damaging effects of ultraviolet (UV) radiation (Brenner and Hearing 2008). The visual properties of melanins allow them to fulfill secondary functions such as concealment and signaling of individual quality in intraspecific communication (Guindre-Parker and Love 2014). In recent years, evolutionary and ecological studies of pigmentation have greatly benefited from considering that vertebrates synthesize two primary forms of melanins that have different visual properties: eumelanins, polymers of indole units, and pheomelanins, oligomers of sulfur-containing heterocycles (e.g., Lindgren et al. 2014). Melanin diversity, however, is actually greater than these two types. Evolutionary ecologists have overlooked this chemical diversity, but considering melanin heterogeneity might help in understanding the physiological consequences of being pigmented, the adaptive benefits of pigmentation patterns, and, ultimately, the evolution of the most common color phenotypes in animals. This is because the different chemical forms of melanins have different physical properties and are synthesized under different physiological conditions.

The process of melanin synthesis that occurs in melanocytes (i.e., melanogenesis) should be considered an oxidative process, as it basically consists of the oxidation of orthodiphenols to orthoquinones to achieve a polymerization of the subunits that form the large pigment molecules. This biosynthetic process generates reactive oxygen species (ROS) and other oxidative subproducts that are potentially toxic to melanocytes. In fact, melanogenesis takes place in specialized suborganelles called melanosomes to avoid cytotoxicity in the cytosol (Borovanský and Riley 2011). Once melanin polymers are formed and transferred to their final destinations, such as keratinocytes or integumental structures including hairs or feathers, melanin has some protective properties against ROS, toxic metals, and xenobiotics, but the capacity of melanins to scavenge these species is limited (McGraw 2005). Some of the genes that control melanogenesis are also involved in cellular antioxidant responses, but a link between melanins and these responses would be mediated by pleiotropic effects and not by a direct antioxidant activity of melanins (Ducrest et al. 2008). Therefore, the direct physiological consequences of melanogenesis are mainly oxidative; hence, it is this oxidative process that is likely to mediate the main evolutionary consequences of melanization. Here we focus on the oxidative conditions under which different melanin forms are synthesized and the potential evolutionary implications derived from them.

Differential Costs of Eumelanin Forms

The indole units of eumelanin, which are composed of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) moieties, result from the decarboxylative or nondecarboxylative rearrangement of dopachrome, a product derived from dopaquinone cyclization. Whether a decarboxylative or nondecarboxylative process occurs depends on microenvironmental conditions (fig. 1). Moreover, once these indolic orthodiphenols (o-diphenols) are formed, they are further oxidized to the corresponding orthoquinones (o-quinones): 5,6-indolequinone (IQ) and indole-2-carboxylic acid-5,6-quinone

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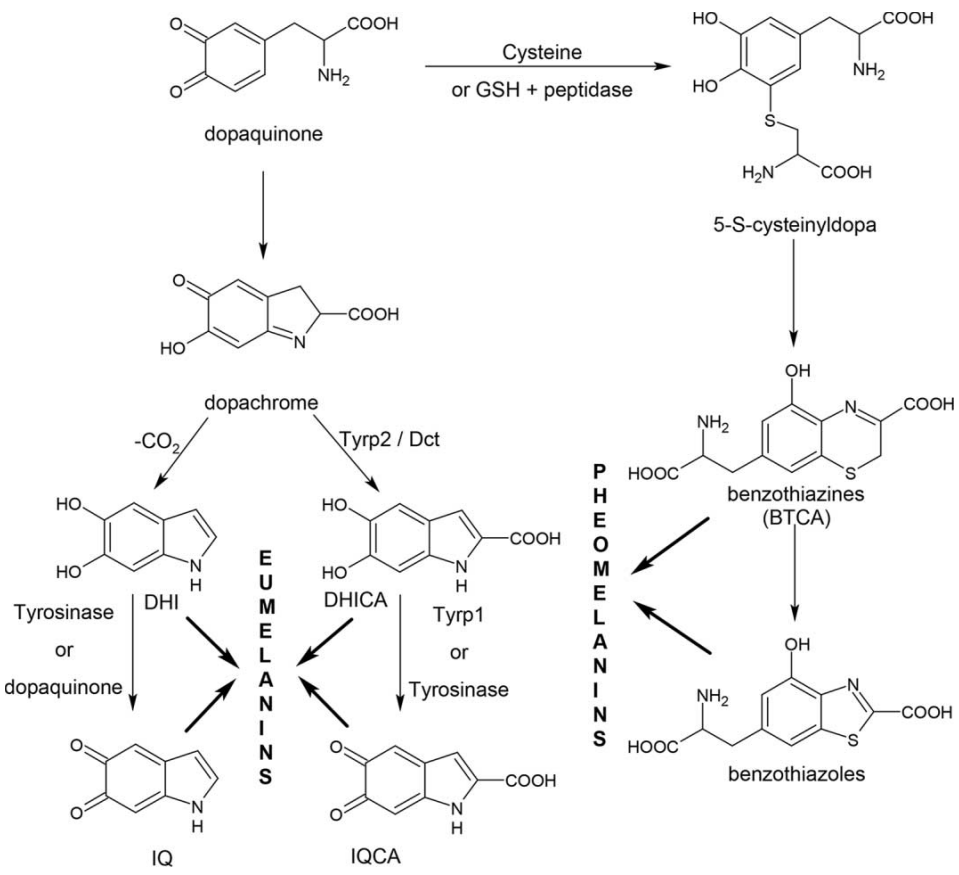

Figure 1. Outline of the melanogenesis pathway. The process starts with the oxidation of the amino acid tyrosine to dopaquinone, which in the presence of certain levels of sulfhydryl compounds such as cysteine or glutathione (GSH) follows the pheomelanogenesis route, whereas in the absence of sulfhydryl compounds dopaquinone follows the eumelanogenesis route.

(IQCA), respectively. The mixture of o-diphenols and o-quinones undergoes further cross-linking reactions, leading to polymerization and, finally, formation of eumelanin (d'Ischia et al. 2009).

What is relevant for evolutionary ecology is the fact that a significant amount of cytotoxic species, including ROS such as superoxide and hydrogen peroxide, are produced during the final stages of melanogenesis downstream of dopachrome (fig. 1). In those reactions, the amount of ROS generated is much greater in the decarboxylative route to DHI/IQ than in the case of the other route through the pair DHICA/IQCA. In fact, melanocytes with no activity of the enzymes Tyrp1 and Tyrp2, which are involved in the DHICA/IQCA route, have lower survival (Hearing 1993; Urabe et al. 1994; Jiang et al. 2010). Further, DHI/IQ polymerization occurs by coupling the indole monomers mainly through 2,4′- and 2,7′-bondings. In contrast, the oxidative coupling of DHICA/IQCA leads to 4,4′-biindolyl, 4,7′-biindolyl, and other minor dimers due to the influence of the carboxyl group at the 2 position of the indole ring, which limits the range of reactive sites available for oxidative coupling (Aroca et al. 1992; Ito and Wakamatsu 2008). As a consequence, the DHI/IQ pathway rapidly leads to large branched polymers that absorb light uniformly across a wide range of wavelengths, while the DHICA/IQCA pathway may lead to smaller linear polymers that exhibit distinct absorbance peaks (Panzella et al. 2013). Thus, producing eumelanins with a high proportion of DHI/IQ implies greater final protection against UV radiation than producing eumelanins with a high proportion of DHICA/IQCA but more ROS production during melanogenesis and therefore more cytotoxicity and less pigment-producing melanocytes (Urabe et al. 1994).

Thus, the different proportions of DHI and DHICA and their corresponding o-quinones give rise to different color phenotypes on which selective pressures can act, as evidenced by three mouse models. The eumelanogenesis of black mice is regulated by three enzymes: tyrosinase and two tyrosinase-related proteins, dopachrome tautomerase (Tyrp2/Dct) and DHICA oxidase (Tyrp1; Urabe et al. 1994; Ito and Wakamatsu 2008). Brown mice are mutants at Tyrp1 and thus produce eumelanin with a different DHI/DHICA ratio than black mice (Jiménez-Cervantes et al. 1994). Slaty (gray) mice are mutants at Tyrp2 and thus produce eumelanin that almost lacks indole-carboxylated units (fig. 1; Aroca et al. 1992; Ito and Wakamatsu 2011). The gray color of slaty mice also results from a low survival and hence a low density of epidermal melanocytes (Urabe et al.

1994). Although gray and other melanin-based color phenotypes can appear for reasons other than the blocking of the DHICA/IQCA route, the hair coloration of these three mouse models indicates that regulating the relative amount of DHI/IQ versus DHICA/IQCA produced during eumelanogenesis contributes to generating different colors. The situation involving the synthesis of benzothiazines versus benzothiazoles during pheomelanogenesis seems to be similar, although a parallel set of mutant mouse models is not available (see below).

Differential Costs of Pheomelanin Forms

Pheomelanogenesis, which diverges from eumelanogenesis at the precursor, dopaquinone (Ito and Wakamatsu 2008), also entails considerable ROS production but after the pheomelanins are formed. Indeed, exposure to energetic radiation, such as UV radiation, causes pheomelanin to produce ROS. This ROS production, however, is reduced in the benzothiazole moiety as compared to the benzothiazine moiety due to the rather stable nature of the former (Wakamatsu et al. 2009). Hence, pheomelanins with higher relative contents of benzothiazoles are less prooxidant under radiation exposure (Galván et al. 2014 and cited references). The analysis of benzothiazine/benzothiazole ratios in different biological tissues gives information about the color phenotypes generated by the different pheomelanin moieties. For example, the hair of recessive yellow mice that exhibit a pale orange coloration has a higher benzothiazine/benzothiazole ratio than red chicken feathers and red human hair (Wakamatsu et al. 2009), suggesting that benzothiazines contribute to generating pale orange colors while benzothiazole contributes to generating darker orange colors. Therefore, pale (yellowish) pheomelanins would be more toxic than dark orange (chestnut) pheomelanins.

Evolutionary Implications of Heterogeneity in Melanin Chemistry

Given the key role of oxidative stress (i.e., when ROS production exceeds the availability of antioxidant compounds) as a determinant of life history strategies (Metcalfe and Alonso-Alvarez 2010), the type and subtype of the melanin that is produced for pigmentation are expected to affect the evolution of life histories. In the case of eumelanogenesis, ROS production is higher, and melanocytes exhibit reduced survivorship when the DHICA/IQCA pathway is blocked and the DHI/IQ pathway predominates. However, the highly branched eumelanins formed through the DHI/IQ pathway may have a greater capacity to protect organisms again UV radiation (Tran et al. 2006). This may represent an evolutionary trade-off. Thus, natural selection may favor the evolution of color phenotypes resulting from both the DHI/IQ and DHICA/IQCA routes under environmental conditions or during physiological processes that produce high levels of oxidative stress so that endogenous ROS production is minimized. In contrast, natural selection may favor the evolution of color phenotypes resulting from the blocking of the DHICA/IQCA route when the need for UV protection is higher than the need for protection from other sources of oxidative stress. This would mean that organisms exposed to high UV radiation may be selected to produce highly branched eumelanins through the DHI/IQ pathway at relatively high rates, despite associated oxidative costs. However, this may in turn hinder their ability to cope with further oxidative challenges. At the same time, eumelanin-based traits resulting from both the DHI/IQ and DHICA/IQCA routes would be the most costly phenotypes under high UV exposure and thus have a greater potential to evolve as honest signals of genotypic quality (i.e., handicaps) for intraspecific communication under these conditions (Galván and Alonso-Alvarez 2009). In contrast, eumelanin-based traits resulting from the blocking of the DHICA/IQCA route would have a greater potential to evolve as honest signals under conditions of high environmental oxidative stress caused by other factors.

Regarding pheomelanins, there is increasing evidence that pheomelanin production represents a cost under environmental conditions or physiological processes that require a high level of protection against ROS production, and it has recently been shown that a shift toward the production of pheomelanins with a higher benzothiazole/benzothiazine ratio facilitates the acclimation of birds to relatively high exposure to ionizing radiation, which produces oxidative stress (Galván et al. 2014). Pheomelanin-based colors confer camouflage or concealment and act as signals of individual quality, but certainly not all pheomelanin-based color traits are involved in concealment or signaling. The only adaptive benefit so far proposed for pheomelanins that can explain their evolution despite its costs (consumption of cysteine during pheomelanogenesis and phototoxicity of pheomelanin polymers) is the removal of excess cysteine under low levels of environmental oxidative stress (Galván et al. 2012). However, nothing is known about possible differences between benzothiazine and benzothiazole moieties regarding this or other benefits. It will be very valuable to account for this information in the future.

Understanding the existence of two main chemical forms of melanins (eumelanin and pheomelanin) and that pheomelanogenesis represents a consumption of a key antioxidant resource has provided evolutionary ecology with useful cues to get insights into the evolutionary consequences of animal pigmentation. Indeed, pheomelanin synthesis is more costly in antioxidant terms than eumelanin, because pheomelanogenesis occurs when cysteine is present over certain levels in melanocytes. Thus, pheomelanogenesis represents a consumption of glutathione, the main intracellular antioxidant and physiological reservoir of cysteine, while cysteine is not consumed during eumelanogenesis (Ito and Wakamatsu 2008). This has led to the realization that producing eumelanin entails different benefits and costs than producing pheomelanin and that pheomelanogenesis may limit the ability to cope with physiological processes or environmental conditions that generate high levels of oxidative stress (Karell et al. 2011; Roulin et al. 2011; Galván and Møller 2013; Galván et al. 2014). However, the diversity of melanins is more than just eumelanin and pheomelanin. Now it appears imperative for evo-

lutionary ecologists to conduct chemical analyses of melanins in animal integuments to determine which color phenotypes generate the distinct forms of melanins in different species. This article represents the first attempt to view the entire chemical diversity of melanins from an evolutionary perspective and opens a completely new perspective on animal coloration and its influence on life history evolution.

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