Insects synthesize pheomelanin

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Dear Editor,

Melanins are the most extended biological pigments, consisting of polymers of indole units (eumelanin) or oligomers of sulfur-containing heterocycles (pheomelanin) (Ito et al., 2011). However, knowledge on the chemistry of melanins is virtually limited to those synthesized by homeotherm vertebrates and more specifically by humans and mice (Ito and Wakamatsu, 2003). The lack of information on poikilotherm melanins is especially evident for pheomelanin, which up to the last 3 years had not been reported in reptiles (Roulin et al., 2013) and amphibians (Wolnicka-Glubisz et al., 2012). Among invertebrate animals, pheomelanin has only been found in a mollusk (Speiser et al., 2014), although there is an unpublished report in butterfly wings (K. Wakamatsu, S. Ito and P.B. Koch). Pheomelanin is associated with increased risk of melanoma in the epidermis (Mitra et al., 2012) and to Parkinson's disease in the brain (Spencer et al., 1998). Finding pheomelanin and understanding its composition in invertebrates may thus allow finding new animal models for these diseases.

Pheomelanin confers chestnut or yellowish colors. We therefore suspected that the colors expressed by some grasshoppers (Insecta: Orthoptera: Caelifera) may be produced at least partly by pheomelanin and aimed to obtain the chemical characterization of these pigments. To this end, we analyzed different specimens of the grasshopper *Sphingonotus azurescens* ranging in color from blackish-gray to reddish-brown (Figure S1).

We first used Raman spectroscopy to search for specific signal of eumelanin and pheomelanin (Galván et al., 2013). We used a Thermo Fisher DXR confocal dispersive Raman microscope (Thermo Fisher Scientific, Madison, WI, USA) with a point-and-shoot Raman capability of 1 μm spatial resolution and using an excitation laser source at 780 nm. The laser beam was focused at two random points of the dorsal part of grasshoppers' cuticle, thus obtaining two Raman measurements from each specimen. Two Raman spectra obtained from a blackish grasshopper showed two distinctive bands at about 1380 and 1580 cm^{-1} resembling the D and G bands characteristic of disordered graphite and absence of signal in the 1750–2500 cm^{-1} region (Figure S1). This Raman spectrum is characteristic of eumelanin (Galván et al., 2013). By contrast, the Raman spectra obtained from a reddish grasshopper showed wide Raman bands at about 500, 1490, and 2000 cm^{-1} characteristic of pheomelanin (Galván et al., 2013; Figure S1). This is indicative of the presence of pheomelanin in the grasshoppers' cuticle.

We next investigated the chemical origin of the pheomelanin found. For this, we analyzed the grasshopper specimens used for Raman analyses and eight additional specimens by high-performance liquid chromatography (HPLC) for the detection of specific degradation products of melanins derived from 3,4-dihydroxyphenylalanine (DOPA) and from dopamine (DA). The specimens were prepared for HPLC analyses by suspending 1 mg of the cuticle of grasshoppers in 100 μl of water. We used 4-amino-3-hydroxyphenylalanine (4-AHP) obtained by reductively hydrolyzing 5-S-cysteinyl-(cys)-DOPA-derived pheomelanin with hydriodic acid (HI) and pyrrole-2,3,5-tricarboxylic acid (PTCA) by oxidizing 5,6-dihydroxyindole-2-carboxylic acid (DHICA)-derived eumelanin with hydrogen peroxide (H_2O_2). We also used 4-amino-3-hydroxyphenylethylamine (4-AHPEA) as the 5-S-cys-DA-derived pheomelanin marker obtained by hydrolyzing with HI (Wakamatsu et al., 2012) and pyrrole-2,3-dicarboxylic acid (PDCA) as the 5,6-dihydroxyindole (DHI)-derived eumelanin marker by oxidizing with H_2O_2 (Wakamatsu et al., 2012). Additionally, we measured two other markers of cys-DA-derived pheomelanin, thiazole-2,4,5-tricarboxylic acid (TTCA), and thiazole-4,5-dicarboxylic acid (TDCA), but the latter could not be detected due to interference by peaks of unknown impurities during the HPLC analyses. It must be noted that conversion factors between degradative markers and levels of melanin polymers are unknown in insects, so that only comparisons between levels of markers, and not between actual melanin levels, can be performed.

All results from the HPLC analyses are shown in Table 1. We found significant amounts of both melanin forms derived from DOPA in all specimens. The levels of PTCA, generated from DHICA-derived eumelanin, were higher than those of 4-AHP, generated from 5-S-cys-DOPA-pheomelanin (4-AHP: PTCA ratio: mean ± SE: 0.6 ± 0.1, range: 0.3–1.0, paired t test: $t = 4.68$, $df = 9$, $P = 0.001$), but lower than the levels of TTCA, generated

Table 1. Results of chemical analyses of melanins in grasshoppers. Absolute values, expressed as nanograms per milligram of insect cuticle, of degradative products of melanins derived from DOPA and dopamine (DA) as calculated by HPLC analysis of 10 specimens of the grasshopper *Sphingonotus azurescens* are reported. The ratios between different degradative markers are also shown. PTCA and PDCA are typical degradation products from DOPA-eumelanin and DA-eumelanin, respectively. TTCA is a degradation product from both cys-DOPA-pheomelanin and cys-DA-pheomelanin. 4-AHP and 4-AHPEA are degradation products from cys-DOPA-pheomelanin and cys-DA-pheomelanin, respectively. Degradation products from grasshopper specimens #1–10 were obtained in a single determination. Specimens #1–3 were reddish-brown (Figure S1A) and specimens #4–10 were blackish-gray (Figure S1B). Ratios between degradative markers of human neuromelanin (NM) are also shown for comparative purposes. Degradation product levels from NM were taken from Zecca et al. (2008)

	H_2O_2 oxidation			HI hydrolysis							
Specimen #	PTCA (ng/mg)	PDCA (ng/mg)	TTCA (ng/mg)	4-AHP (ng/mg)	4-AHPEA (ng/mg)	4-AHP: PTCA	TTCA: PTCA	4-AHPEA: PDCA	4-AHPEA: 4-AHP	4-AHPEA: TTCA	PDCA: PTCA
1	24.6	n.d.	n.d.	70.9	90.7	2.88	–	–	1.28	–	–
2	12.8	11.7	22.6	20.8	75.7	1.63	1.77	6.47	3.64	3.35	0.91
3	9.5	8.2	34.5	34.9	72.1	3.67	3.63	8.79	2.07	2.09	0.86
4	11.7	15.3	42	21.4	80.5	1.83	3.59	5.26	3.76	1.92	1.31
5	38.7	16.4	118	86.8	239.1	2.24	3.05	14.58	2.75	2.03	0.42
6	16.3	19.9	50.7	83.2	84.7	5.10	3.11	4.26	1.02	1.67	1.22
7	13.8	5.3	75.5	26.9	81.7	1.95	5.47	15.42	3.04	1.08	0.38
8	17.2	8.8	44.2	57.5	61.9	3.34	2.57	7.03	1.08	1.40	0.51
9	27.6	86.7	90.8	78.3	112.9	2.84	3.29	1.30	1.44	1.24	3.14
10	30.7	134	114	79.5	120.2	2.59	3.71	0.90	1.51	1.05	4.36
Mean ± SE	20.29 ± 3.04	34.03 ± 15.03	65.81 ± 11.71	56.02 ± 8.62	101.95 ± 16.24	2.81 ± 0.33	3.35 ± 0.33	7.11 ± 1.71	2.16 ± 0.33	1.76 ± 0.24	1.46 ± 0.46
NM	62	106	717	18	454	0.29	11.6	4.28	25.2	0.63	1.71

n.d., not detected due to overlap with unknown compounds during HPLC analyses.

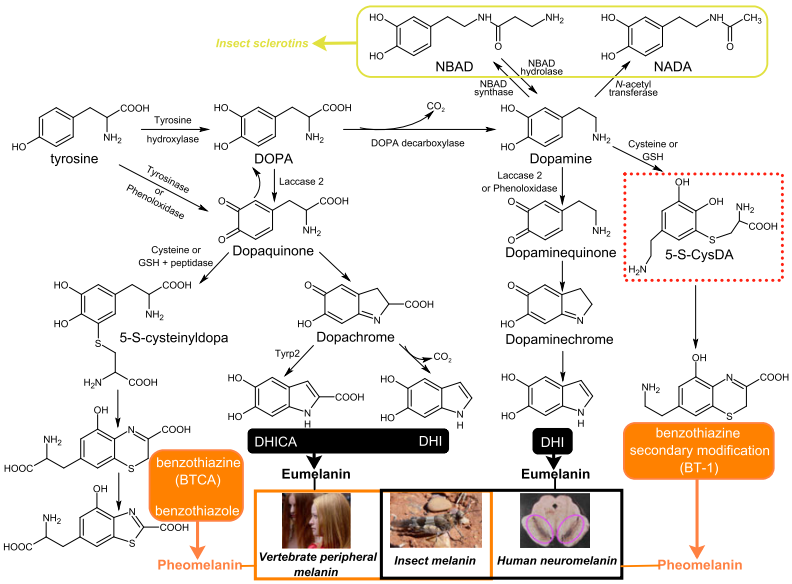

Figure 1. Schematic representation of the biochemical pathway leading to the synthesis of vertebrate and insect melanins from DOPA and from DA and to the synthesis of insect sclerotins from dopamine. Our study provides the first evidence of the formation of pheomelanin in insects, including the route from DA, which coincides with the formation of the pheomelanic moiety of human neuromelanin. The neurotoxic intermediate cys-DA is marked in red. Arrows with no enzymes associated represent non-enzymatic reactions.

from cys-DA-derived pheomelanin (TTCA: PTCA ratio: 3.3 ± 0.3, range: 1.8–5.5, t = 5.25, *df* = 8, P < 0.001).

We also found amounts of other melanin forms derived from DA. The levels of the marker 4-AHPEA, generated from 5-S-cys-DA-pheomelanin, were significantly higher than those of the marker PDCA, generated from DHI-eumelanin (4-AHPEA: PDCA ratio: 7.1 ± 1.7, range: 0.9–15.4, t = 3.24, *df* = 8, P = 0.012). Interestingly, the levels of 4-AHPEA were significantly higher than those of 4-AHP, generated from 5-S-cys-DOPA-pheomelanin (4-AHPEA: 4-AHP ratio: 2.1 ± 0.3, range: 1.0–3.8, t = 3.42, *df* = 9, P = 0.008). There were no differences, however, between the levels of PDCA, generated from DA-eumelanin, and those of PTCA, generated from DOPA-eumelanin (PDCA: PTCA ratio: 1.5 ± 0.4, range: 0.4–4.4, t = 1.06, *df* = 8, P = 0.321).

Our finding confirms that the presence of pheomelanin is not limited to vertebrate animals and also occurs in insects. There is a recent published report of pheomelanin in a mollusk (Speiser et al., 2014), but our study extends the presence of pheomelanin to the group of most abundant animals (Insecta). The prevalence of pheomelanin among animal groups has not been exhaustively investigated, but these findings suggest that this pigment may be widespread in the animal kingdom, as opposed to what was previously thought (Galván et al., 2012; Ito and Wakamatsu, 2003).

Overall, our chemical degradation results indicate that grasshoppers produce rather complex, mixed-type melanins arising from DOPA, cys-DOPA, DA, and cys-DA. It is interesting that most pheomelanin in grasshoppers derives from DA as indicated by a mean 4-AHPEA/4-AHP ratio of 2.16 (Table 1). This is not surprising, as insects synthesize eumelanin by oxidizing DA to DA *ortho*-quinones that are further oxidized to finally generate indolequinones, the precursors of DHI (Sugumaran, 2002) (Figure 1). However, it had not previously been found that DA also leads to the production of pheomelanin in insects.

Almost all vertebrate melanins (mostly peripheral, produced in epidermal melanocytes) are synthesized from DOPA. The exception is neuromelanin (NM), which is composed of mixed melanins derived from both DOPA and DA in catecholaminergic neurons (Napolitano et al., 2011; Zecca et al., 2008) (Figure 1). Our chemical analyses of grasshoppers also resulted in mixed melanins from DOPA and DA as in NM, but the proportions of all degradative markers differed from those reported for human NM. Specifically, the ratios TTCA:PDCA, 4-AHPEA:PDCA, TTCA:4-AHPEA, PDCA:PTCA, and TTCA:PDCA were not close to the ratios reported for natural NM (Table 1). Interestingly, however, some of these ratios were similar to those corresponding to a synthetic NM [DA+cys (1:0.5)-melanin] (Wakamatsu et al., 2012). Thus, for example, the ratio PDCA:PTCA in the grasshopper specimens #9 and #10 (Table 1) was close to the corresponding ratio in the synthetic NM (3.47; Wakamatsu et al., 2012). It is also worth mentioning that the ratio PDCA:PTCA in grasshoppers indicates that the role of dopachrome isomerase favors the production of DHI instead of DHICA. Future studies should investigate the potential similarity between insect pheomelanin and the pheomelanic core of NM (Bush et al., 2006). It will also be interesting to investigate possible roles of pheomelanin in insect defense mechanisms and cuticle sclerotization.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Raman spectra of melanins obtained from the cuticle of grasshoppers.