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Xanthurenic acid is a pigment in *Junonia coenia* butterfly wings

Emily Vanessa Daniels*, Robert Dale Reed

Department of Ecology and Evolutionary Biology, 321 Steinhaus Hall, University of California, Irvine, CA 92697-2525, USA

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1. Subject and source

The buckeye butterfly *Junonia coenia* is a model system for studies of butterfly wing pattern development (Kodandaramaiah, 2009; Kusaba and Otaki, 2009,Nijhout and Koch, 1991; Weatherbee et al., 1999). In the eastern United States *J. coenia* shows a distinct seasonal polyphenism where Spring/Summer morphs (*linea*) have pale brown wing coloration and Autumn/Winter morphs (*rosa*) show darker red wing coloration. We kept a laboratory colony of *J. coenia* originating from Durham County, North Carolina (35°59′19″ N, 78°54′26″ W) as provided by Fred Nijhout. Butterflies were reared in incubators and fed a standard artificial diet (Nijhout, 1980). Two sets of conditions known to induce alternative seasonal color morphs were applied (Smith, 1991): 21 °C with 8 h light for short/cool day conditions and 27 °C with 16 h light for long/warm day conditions, producing red and pale brown ventral wing surfaces respectively. The wings of mature adults of both the red and pale brown morphs were examined.

2. Previous work

It was previously shown that *J. coenia* pupae injected with the radiolabeled ommochrome pigment precursors tryptophan and 3-hydroxykunurenine show strong incorporation in red and red-brown wing pattern elements (Nijhout and Koch, 1991; Koch, 1993), thus suggesting that the red pigmentation in *J. coenia* is largely due to pigments in the tryptophan–ommochrome pathway. Nijhout (1997) used thin layer chromatography (TLC) to identify three major presumptive ommochrome pigments in *J. coenia* wings: an orange-yellow pigment, a red-brown pigment, and a red pigment. The two red pigments were found to be abundant in the dark red morph of *J. coenia*, while the orange–yellow pigment was found to be abundant in the pale brown morph. Based mostly on absorbance spectra and TLC migration rates it was proposed that the red and red–brown pigments were dihydro-xanthommatin and ommatin-D respectively, and the yellow–orange pigment was xanthommatin. Unfortunately, however, there were no mass spectrometry (MS) data available to rigorously verify the identities of either the

* Corresponding author. Tel.: +1 949 769 1156; fax: +1 949 824 2181.
E-mail address: edaniels@uci.edu (E.V. Daniels).

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standards or samples used in any of this work, so the actual identities of the major *J. coenia* ommochrome pigments remain an open question.

3. Present study

Ommochrome pigments were extracted from whole hindwings of both the color morphs in 1 ml acidified methanol (0.5% HCl) according to an established protocol (Nijhout, 1997). To maximize stability wing extracts were kept in darkness at 4 °C prior to analysis. An aliquot of each extract was used for positive ionization mass Spectroscopy (Waters, LCT Premier) to establish the masses of compounds in the extract. Portions of the extracts were also subjected to liquid chromatography–mass spectrometry (LC/MS), a diode array detector (DAD), and tandem mass spectrometry (MS/MS) (Waters, Quattro Premier XE – with Waters Acquity UPLC C-18 column dimensions 50 mm × 2.1 mm with 1.7 μ particles) running on a linear gradient protocol for 15 min to establish the retention times of the components in the extract. The primary solvent was water with 0.2% acetic acid, with a gradient of 0.5%–95% acetonitrile in 0.2% acetic acid. Masses, spectra, and retention times were compared to analytical grade standards of compounds present in the tryptophan–ommochrome pigment biosynthesis pathway dissolved in the same solvent as the wing extracts. Standards for tryptophan, kynurenine, kynurenic acid, kynurine, anthranilic acid, 3-hydroxykynurenine, xanthurenic acid, and 3-hydroxy-anthranilic acid were obtained from Sigma Aldrich.

J. coenia wing extracts from both red and pale brown color morphs contained a compound matching the mass (205.0), absorbance spectrum ($\lambda_{max} = 244$ nm, 342 nm), and HPLC retention time (2.8 min) of the pure xanthurenic acid standard. Furthermore, when MS/MS was conducted on the xanthurenic acid standard and wing extracts using collision energy of 25 V the same three mass peaks of 132, 160, and 188 were observed in every case. Together our data provide very strong support for xanthurenic acid indeed being present in the wing extracts of *J. coenia* butterflies of both color forms. Surprisingly none of our wing extracts contained compounds with masses matching any form of xanthommatin, dihydro-xanthommatin, ommatin-D, or any other known pigment in the tryptophan-ommochrome pathway.

4. Chemotaxonomic significance

Xanthurenic acid has previously been identified as a pigment in various hemimetabolous and holometablous insects (Linzen, 1974), including in the grashopper *Schistocerca gregaria* (Pinamonti et al., 1964), in the wings of the Indian meal moth *Plodia interpunctella* (Mohlmann, 1958), the domesticated silkmoth *Bombyx mori* (Inagami, 1958), the parasitic wasp *Habrobracon juglandis* (Leibenguth, 1965), the vinegar fly *Drosophila melanogaster* (Umebachi and Tsuchitani, 1955; Wessing and Eichelberg, 1968), the house fly *Musca domestica* (Colombo and Pinamonti, 1967; Laudani and Grigolo, 1968), and in flower spiders (Riou and Christidès, 2010; Insausti and Casas, 2008). This is the first report of this molecule serving as a butterfly wing pigment.

Our finding suggests that the tryptophan metabolism pathway used during *J. coenia* wing development may be much different than previously supposed. Previous authors have drawn on work on xanthommatin synthesis in *Drosophila* eyes to model how ommochromes may be synthesized in nymphalid butterfly wings (Reed and Nagy, 2005; Reed et al., 2008; Ferguson and Jiggins, 2009). In contrast to this, the presence of xanthurenic acid in butterfly wings would require an alternative biosynthetic branch of the tryptophan–ommochrome synthesis pathway (Linzen, 1974). This significantly expands our notion of wing pigment diversity, as all previously proposed nymphalid ommochromes we are aware of are derived from the xanthommatin/ommin synthetic branch. The synthesis of xanthurenic acid from 3-hydroxykynuernine was recently confirmed in the mosquito *Aedes aegypti*, and the enzymes involved in each step were identified for that species (Han et al., 2007) – this case study may serve as a useful starting point for understanding pigment synthesis in *J. coenia.*

Another interesting result from our MS work was that xanthurenic acid was the only compound we identified that matched a known pigment in the tryptophan–ommochrome pathway. This was despite the fact that we observed the presence of numerous compounds of various molecular weights in the wing extracts. Although more work is required before we can identify the molecular identities of these novel compounds and/or definitively rule out the presence of xanthommatin, dihydro-xanthommatin, or ommatin-D in *J. coenia* wings, our results raise the possibility that a rigorous reconsideration of *J. coenia* wing pigmentation is warranted.

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Appendix A. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.bse.2012.04.025.

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