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URIC ACID DEPOSITION IN LARVAL INTEGUMENT OF BLACK SWALLOWTAILS AND SPECULATION ON ITS POSSIBLE FUNCTIONS

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ABSTRACT. From the first through third instar, larvae of the black swallowtail, *Papilio polyxenes* Fabr., display a distinctive color pattern characterized by an irregular circle of white pigment on the dorsum. This white spot is surrounded by brown pigmentation, creating the impression of a bird dropping. It has long been assumed that this color pattern evolved as a defense mechanism against avian predators. We report here that the source of the white color is accumulated uric acid. Although uric acid has traditionally been viewed as an excretory product, it also can act in biological systems as a powerful antioxidant. Thus, the possibility exists that the white spot serves a protective function not only against predators in a high fidelity mimicry system, but also against oxidative stress generated by the phototoxic allelochemicals that characterize most hostplants of *Papilio* species.

Additional key words: *Papilio polyxenes*, bird dropping, furanocoumarin.

Early instars of a range of Lepidoptera species are brownish in color with a white saddle traversing the middle of the abdomen; this color pattern, from the human perspective, bears a remarkable resemblance to a bird dropping and thus has been characterized as an example of homotypism, or protective resemblance to an object considered inedible by a predator (Edmunds 1974). Although this pattern has been reported in at least one nymphalid genus (and typifies the larvae of the viceroy *Basilarchia archippus archippus* (Cramer), the red-spotted purple *Basilarchia arthemis astyanax* (Fabr.), and the white admiral *Basilarchia arthemis arthemis* (Drury)), it is practically universal among early stages of species in the genus *Papilio* (Lepidoptera: Papilionidae) (Munroe 1961).

Among the many substances contributing to larval lepidopteran pigmentation patterns is uric acid. Although uric acid is the major nitrogenous waste product of terrestrial insects (Cochran 1985), in many species it is retained and deposited in body tissue. This mechanism of dealing with excess nitrogen is known as storage excretion (Wigglesworth 1942, 1965, 1987) and provides a source of pigmentation, particularly in larval stages. Mauchamp and Lafont (1975) demonstrated that most of the uric acid in young *Pieris brassicae* (L.) caterpillars lies in the integument and is accumulated in the fat body before pupation, and Buckner and Newman (1990) determined that uric acid deposition in the integument is principally responsible for the appearance of white stripes that contrast with the green abdominal base color. Uric acid, presumably generated by larval metabolism, also can be found as a pigment in the yellow scales of *Papilio xuthus* L. (Tojo & Yushima 1972) and in the wings of male *Pieris brassicae* (Lafont & Pennetier 1975).

In that birds are uricotelic, producing uric acid as the principal form of waste nitrogen, the white color of bird droppings is due to the presence of uric acid. We examined the integument of larvae of *Papilio polyxenes* Fabr., the black swallowtail butterfly, a species that displays the typical *Papilio* early stage "bird dropping" morphology, in order to determine if in fact the white saddle results from the accumulation of uric acid, the substance that provides the model for the mimetic resemblance. We also examined the integument of ultimate (fifth) instars, to determine if uric acid contributes to color patterns in mature larvae as well, and in adult male butterflies, to ascertain whether sequestered uric acid is retained through metamorphosis.

MATERIALS AND METHODS

The eastern black swallowtail butterfly, *Papilio polyxenes asterius* Stoll., is found throughout eastern North America, ranging from southern Canada to Florida; it also occurs west along the eastern Rockies into northern Mexico (Opler and Krizek 1984). The larval stages feed almost entirely on herbaceous representatives of the families Apiaceae and, to a more limited extent, Rutaceae, and are found in a variety of open habitats. Larval development requires from two to three weeks, depending on temperature and hostplant (Blau 1981). Larvae in the first three stadia are primarily black, with a characteristic white saddle across the dorsal midsection; fourth and fifth instars are greenish-white to green, with black bands running horizontally across each segment, interrupted by a series of yellow to orange spots (Fig. 1). In central Illinois, there are two to three generations each year. Principal natural enemies of the black swallowtail in-

FIG. 1. Third (left) and fifth (right) instar *Papilio polyxenes* caterpillars.

clude spiders, wasps, predaceous bugs in the families Nabidae, Reduviidae, Coreidae, and Pentatomidae (Blau 1981, Feeny et al. 1985), ants, and possibly birds. Caterpillars defend themselves with an eversible osmeterial gland, the constituents of which change developmentally from primarily mono- and sesqui-terpenes in early instars to aliphatic acids and their esters in fourth and fifth instars (Berenbaum et al. 1992).

Gravid female *P. polyxenes* were collected in Champaign County, Illinois, and allowed to oviposit on foliage of parsley, *Petroselinum crispum* (Mill.); eggs collected in this manner were used to found a colony from which larvae were taken for experimental use. Caterpillars were reared on potted parsley or on parsnip, *Pastinaca sativa* L., plants in a greenhouse at 27°C (day): 21°C (night) under a 16L:8D photoperiod. As caterpillars reached either third or fifth instar, they were collected for chemical analysis. Male adults collected earlier from a laboratory colony initiated with wild-caught butterflies from Champaign County, Illinois, were stored at –80°C prior to chemical analysis.

Prior to all larval tissue collections, we removed the gut and Malpighian tubules. Epidermis of the abdomen of the third instar was divided into portions corresponding to the white saddle area and the remaining brown portion; each portion was analyzed separately. The final instar was divided according to apparent integument coloration. Black bands, green ground color, and yellow spots were cut out with iridectomy scissors and analyzed separately. The wings of the males were divided into black parts and yellow spots by cutting out the spots and each tissue type was analyzed separately. All tissues were collected directly into dry ice-chilled microcentrifuge tubes (1.8 ml) and kept in a freezer at –80°C prior to the uric acid assay.

We homogenized tissue samples with a tissue tearor (Biospec Products, Inc., Bartlesville, Oklahoma) and used a chloroform rinse to free the solution of lipophilic compounds. The integument was eluted in lithium carbonate (1%) to dissolve uric acid and after centrifugation (12,200 g) an aliquot of the supernatant was used for uric acid determination (Van Handel 1975). Each aliquot was brought up to a volume of 2 ml with distilled water. To each sample we added 1 ml of reagent (copper sulfate 0.05%, glycine 1.6%, sodium carbonate 4%) and 0.05 ml neocuproine reagent (Sigma, St. Louis, Missouri). Optical density was determined at 450 nm wavelength (Perkin Elmer Lambda 3B spectrophotometer). Because ascorbic acid can interfere in the uric acid assay (25 µg shows the same optical density as 0.9 µg uric acid), we corrected uric acid readings according to the ascorbic acid content of the sample (Omaye et al. 1979). To quantify ascorbic acid, 0.5 ml of supernatant was added to 0.5 ml of ice-cold 10% trichloroacetic acid, mixed thoroughly, and centrifuged for 5 minutes (12,200 g); 0.5 ml of supernatant was mixed with 0.1 ml of DTC (thiourea, $CuSO_4.5H_20$, 2,4-dinitrophenylhydrazine in 9N H_2SO_4: Omaye et al. 1979) and incubated for 3 hours at 37°C, to form the 2, 4-dinitrophenylhydrazone. After incubation, the test tube was removed from the water bath and placed into ice water; 0.75 ml of ice-cold H_2SO_4 (65%) was added and the solution mixed well. Solutions stood at room temperature for 30 more minutes, after which time we measured the absorbance at 520 nm.

RESULTS AND DISCUSSION

Integument from ten third instars and nine fifth instars and wing scales from three adult male butterflies were analyzed for their uric acid content and distribution. All parts of the integument of third instar *P. polyxenes* contain measurable amounts of uric acid (Table 1). The white saddle, however, contains over

TABLE 1. Distribution of uric acid in the variously colored integuments of third and fifth instar and male wings of *Papilio polyxenes*. Values are given as the mean (standard deviation).

Life stage	N	Integument color	Uric acid (g/mg)
Third	10	Brown	57.2 (16.1)
Third	10	White	116.0 (57.6)[a]
Fifth	9	Black	24.9 (6.9)
Fifth	9	Green	49.1 (20.3)[b]
Fifth	9	Yellow	45.5 (9.2)[c]
Male	3	Black	2.2 (0.5)
Male	3	Yellow	15.0 (2.5)[d]

[a] Significantly different from brown integument, paired $t = 3.72$, $p = 0.005$
[b] Significantly different from black integument, paired $t = 3.2$, $p = 0.013$
[c] Significantly different from black integument, paired $t = 5.7$, $p = 0.0001$
[d] Significantly different from black scales, paired $t = 8.6$, $p = 0.01$

twice the amount of uric acid in brown-colored integument (paired t test, $t = 3.72$, $p = 0.005$). Integument that appears light in color in fifth instars also contains significantly greater amounts of uric acid than does adjacent integument that is black in color. Yellow spots contain almost twice as much uric acid ($t = 5.65$, $p = 0.0001$) as black stripes; the green ground color also contains almost twice the uric acid content of the black stripes ($t = 3.19$, $p = 0.013$). The accumulation of uric acid in the yellow wing scales in male butterflies is sevenfold higher than the amount of uric acid in the black wing scales ($t = 8.6$, $p = 0.01$). Over all life stages examined, uric acid content is highest in the white saddle of the third instar.

To a large extent, caterpillars rely on coloration to avoid visually orienting natural enemies. Although some species rely on crypsis (matching their background) and concealment, many can remain in plain sight by virtue of aposematism and associated unpalatability or by mimetic resemblance to inedible objects or substances. Birds have been identified as important predators of caterpillars in general (Morris 1972, Holmes et al. 1979, Atlegrim 1989, Marquis & Whelan 1994) and the body coloration of many species is thought to reflect selection pressures exerted by avian predation. Resemblance to a bird dropping may be common in that it is likely such a color pattern would be unappetizing to a wide variety of birds. In *P. polyxenes*, the appearance of the white saddle of the "bird dropping" pattern is largely due to the accumulation of uric acid, the same substance that creates the white, shiny appearance of authentic bird droppings. The fidelity of the visual mimetic resemblance to a bird dropping is no doubt enhanced by use of a substance identical to that found in the "model."

Resemblance to a bird dropping is at least in part responsible for the ability of early stage *P. polyxenes* to forage in full view of predators. Such foraging is advantageous in that caterpillars can process food more quickly and efficiently at higher temperatures associated with daylight hours (Ali et al. 1990). Such behavior, however, is not without attendant risks. Exposure to sunlight (particularly ultraviolet wavelengths) can cause oxidative stress; this stress may be exacerbated by the presence of photosensitizing allelochemicals in foliage. The apiaceous hosts of *P. polyxenes* (indeed, of most *Papilio* species—Berenbaum 1983) characteristically possess furanocoumarins, photosensitizers that can cause oxidative damage to DNA (Berenbaum 1991). Although all stages of *P. polyxenes* are capable of rapid and efficient metabolism of these compounds (Harrison et al. submitted), early instars in general have higher relative consumption rates than late instars (Slansky & Scriber 1985) and thus may encounter greater quantities of furanocoumarins relative to their body weight. While melanin in the dark parts of *P. polyxenes* integument may function as a neutral density filter, eliminating photoactivating wavelengths, the white saddle could potentially leave caterpillars vulnerable to ultraviolet light exposure.

Uric acid may be selectively retained by *P. polyxenes*, and *Papilio* species in general, not only because of its color but because of its powerful antioxidant and radical-scavenging properties (Becker 1993). The function of uric acid as an antioxidant in insects has already been established. Souza et al. (1997) observed greatly increased urate concentrations in the hemolymph of *Rhodnius prolixus* following a blood meal and suggested an antioxidant protective function of urate against prooxidant activity generated during the hydrolysis of hemoglobin. In *Drosophila melanogaster*, the antioxidant properties of urate have been demonstrated by the sensitivity of urate-null mutants to experimentally induced oxidative stress (Hilliker et al. 1992). The role of uric acid in epidermis as a protective pigment has also been suggested. In *Anopheles* mosquito larvae, the white dorsal pigmentation may represent protective coloration against solar radiation. Anopheline larvae are confined to shoal biotopes, living at the air/water interface and lying horizontally immediately below the water surface. Under these circumstances, the antioxidant properties of uric acid could effectively reduce possible damage by UV radiation (Benedict et al. 1996). Other antioxidants (particularly antioxidant enzymes) are known to occur in the integument of swallowtails (Lee and Berenbaum 1992), and foliage of swallowtail hostplants has been demonstrated to produce singlet oxygen at the leaf surface (Berenbaum and Larson 1988), which may present particular risks to integumentary tissues.

Thus, the high concentrations of uric acid in the

white saddle of the "bird dropping" morph of young black swallowtail caterpillars may serve multiple purposes—contributing to a compelling mimetic/protective resemblance to an inedible object in the environment while at the same time scavenging free radicals generated by ultraviolet light exposure and ingestion of photosensitizers. Such physiological economy may be enhanced further by the fact that accumulation of uric acid, a waste product generated as a consequence of processing food, does not divert nitrogen away from other physiological needs, as would synthesis of such nitrogenous pigments as pteridines or ommochromes.

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