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Poor sequestration of toxic host plant cardenolides and their rapid loss in the milkweed butterfly *Danaus chrysippus* (Lepidoptera: Nymphalidae: Danainae: Danaini)

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ABSTRACT

Butterflies of the genus *Danaus* are known to sequester toxic cardenolides from milkweed host plants (Apocynaceae). In particular, *Danaus plexippus* efficiently sequesters and stores these compounds, whereas *D. chrysippus*, is considered to poorly sequester cardenolides. To estimate its sequestration capability compared with that of *D. plexippus*, larvae of both species were jointly reared on *Asclepias curassavica* and the major cardenolides of the host plant, calotropin and calactin, were analyzed in adults sampled at different time intervals after eclosion. Both cardenolides were detected in body and wings of *D. plexippus*. Whereas the calotropin-concentration remained constant over a period of 24 days, that of calactin steadily decreased. In the body, but not in the wings of *D. chrysippus*, calactin only was detected in low amounts, which was then almost completely lost during the following 8 days after eclosion, suggesting that in contrast to *D. plexippus*, cardenolides seem to be less important for that butterfly's defence against predators.

1. Introduction

Besides synthesizing a variety of deterrent and toxic compounds for defence, insects also acquire toxicity by sequestering and storing noxious chemicals from their host plants (Whitman et al., 1990; Bell, 2001). As a classical example, the monarch butterfly, *Danaus plexippus*, becomes unpalatable to predators, because its larval host plants, i.e., milkweeds (Apocynaceae), contain toxic cardenolides which are sequestered and retained in the adult stage reducing the susceptibility of adult butterflies to natural predation such as by birds (Brower and Brower, 1964; Parsons, 1965; Reichstein et al., 1968; Roeske et al., 1976; Brower, 1984; Brower et al., 1988; Malcolm and Brower, 1989; Malcolm, 1994; Agrawal et al., 2012; Petschenka and Agrawal, 2015). Tolerance to the toxicity of cardenolides is based on the lack of binding to the extracellular loop in the transmembrane domain M1 of the Na^+, K^+-ATPase alpha-subunit due to the substitution of amino acids at two relevant positions: L111V and N122H (Holzinger et al., 1992; Holzinger and Wink, 1996; Mebs et al., 2000; Dobler et al., 2011; Aardema et al., 2012). In other *Danaus* species such as *D. gilippus, genutia* and *chrysippus* one of the two substitutions only is present: L111V, which increases the *in vitro* resistance of the enzyme to cardenolides, but to a lesser extent than in *D. plexippus* (Petschenka et al., 2013).

Alonso-Meija and Brower (1994) observed that in *D. plexippus* the cardenolide concentration decreases in body and wings with the age of individual butterflies, regardless of the initial amount of the cardenolides or of their chemical structures. Over a period of 25 days, loss rates for cardenolides of more than 60% in the wings and of more than 30% in the abdomen were found to occur. The mechanisms involved in this age-related decline of cardenolides are unknown. As a result of this phenomenon monarch butterflies may change from being unpalatable models to palatable mimics during their lifetime suggesting that freshly eclosed butterflies may serve as noxious models for older individuals, which are suggested to become automimics as they age.

D. chrysippus, the plain tiger or African queen, is widespread in Africa, southern Europe, and Asia (Smith, 2014; Braby et al., 2015). Rothschild et al. (1975) reared larvae of this butterfly from East- and West-Africa on *Calotropis* and *Asclepias* plants and analyzed the adults as well as wild-caught specimens for the presence of cardenolides. They found that *D. chrysippus* is less efficient in sequestering and storing these compounds than *D. plexippus*. Whereas butterflies from East-Africa contained only small amounts

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of cardenolides, those from West-Africa exhibited not even traces. A more recent study on *D. chrysippus* adults caught in Namibia, South-Africa and Moçambique showed that among 75 specimens 4 only contained trace amounts, all others were entirely free of cardenolides (Mebs et al., 2005).

In the present study, larvae of *D. chrysippus* and as control of *D. plexippus* were reared on a shared host plant, *Asclepias curassavica*. After eclosion, butterflies were killed at various time intervals and the presence of the cardenolides, calotropin and calactin, was assayed in body and wings to estimate the capability of the two species to sequester these compounds and to retain the level of these cardenolides over time.

2. Materials and methods

2.1. Butterflies

Larvae of *D. plexippus plexippus* (Spain origin) and *D. chrysippus bataviana* (from Bali, Indonesia) were both reared on *A. curassavica* plants in the laboratory. After eclosion the butterflies (*D. p. plexippus*, 9 males, 3 females; *D. chrysippus bataviana*, 15 males, 13 females, respectively) were kept in a greenhouse at 25 °C and sampled at defined time intervals (up to 24 days), killed in a deep-freezer and were then air dried.

2.2. Analytical procedures

Air dried plant leaves, the body and wings of the butterflies were weighted, ground in a mortar and were extracted with 80% methanol for 5 days at ambient temperature. All extracts were evaporated to dryness at 25 °C in a stream of air. For analysis of cardenolides, thin-layer chromatography (TLC) was performed according to the method of Nelson (1993a) and liquid chromatography linked to time-of-flight mass spectrometry (LC/TOF-MS) was applied as described by Mebs et al. (2012). Among the main fractions matching respective R_f-values in TLC, the two cardenolides, calotropin and calactin, were identified (*m/z*: 533.2745 Da of protonated species; in the extracted ion chromatogram [EIC] the first peak was found to correspond to calotropin, the second to calactin, respectively), two others, i.e., gomphogenin (*m/z*: 391.2400) and gomphosid (*m/z*: 519.2800), represented minor compounds. As reference substances of these compounds are not available, for quantitative analysis the concentration of calotropin and calactin was calculated based on values for digitoxin used as standard compound and are expressed as digitoxin equivalents per mg dry weight.

2.3. Statistical analysis

Analysis for the hypothetical correlation of the calotropin/calactin concentration with age was performed using the nonparametric Spearman's rank correlation test (SPSS version 22, IBM, Ehingen, Germany).

3. Results

By thin-layer chromatography and LC/TOF-MS, calotropin and its diastereomer calactin were identified as major, gomphogenin and gomphosid as minor cardenolides in the leaves of the host plant *A. curassavica*. As expected, both calotropin and calactin were found to be present in the body and wings of *D. plexippus* ($n = 12$, Fig. 1 B, C), confirming the sequestration of these compounds from the larvae's host plant. After eclosion, the mean concentration of calotropin ($n = 3$, expressed as digitoxin equivalents) was found to be three times higher in the wings than in the body (Fig. 2). Likewise, calactin was also present in both compartments and exhibited a two-fold higher concentration in the body than calotropin, whereas in the wings, calactin levels were only slightly higher than in the body. Although the level of calotropin remained stable over a period of 24 days in the body as well as in the wings of the butterflies, the calactin level drastically decreased over time in both compartments. Using the nonparametric Spearman's rank correlation test, a significant correlation of the calactin content with time after eclosion was found in the body of *D. plexippus* ($p = 0.001$), in the wings the correlation was approaching significance ($p = 0.069$), while for calotropin no significant correlations were observed (body: $p = 0.45$; wing: $p = 0.30$).

In contrast, the body of freshly eclosed *D. chrysippus* specimens contained only calactin ($n = 6$, one of them was negative), but about 5 times less than in *D. plexippus*; none of the two cardenolides was detected in the wings (Fig. 1 D). From the fourth to the eighth day after eclosion, most specimens ($n = 15$) were found to be free of calactin (Fig. 2), in six only this compound was detected in low amounts, indicating that *D. chrysippus* poorly sequestered calactin, and that it is rapidly lost thereafter. In the Spearman's rank test, the correlation of calactin levels with time was significant ($p = 0.021$).

No statistically significant differences in cardenolide concentrations were observed between sexes of both species, as shown in Fig. 2.

4. Discussion

The present experiments corroborate earlier findings that *D. plexippus* efficiently sequesters both cardenolides, calactin and calotropin, from its host plant, *A. curassavica,* in body and wings (Mebs et al., 2012). But when larvae of *D. chrysippus* are reared on the same plant, low amounts of calactin only were detected in the body, not in the wings of the butterflies, suggesting poor and selective sequestration of that cardenolide. Moreover, few days after eclosion, calactin was not detectable in most specimens. This cardenolide was most likely lost as the butterflies age.

By feeding larvae of both species with *A. curassavica*, Rothschild et al. (1973, 1975) have shown that *D. plexippus* is a far more efficient sequesterer of cardenolides than *D. chrysippus*. Brower et al. (1975) confirmed the poor sequestration capability of *D. chrysippus*, since only 8 of 50 wild-caught specimens from Ghana exhibited detectable amounts of cardenolides. Mebs et al. (2005) was not able to detect even traces of cardenolides in *D. chrysippus* specimens from southern Africa.

Alonso-Meija and Brower (1994) reported that a few days only after eclosion the concentration of cardenolides in the abdomen and wings of individual *D. plexippus* butterflies decreased logarithmically with time, irrespective of the host plants, *A. humistrata* and *A. syriaca*. However, in the present study a different pattern was observed, when the two main cardenolides, calotropin and calactin sequestered from the host plant *A. curassavica*, were separately evaluated over a similar time period: in *D. plexippus* the calotropin concentration remained constant in the body and wings during 24 days, whereas that of calactin decreased. In contrast, only calactin was detected in the body of freshly eclosed *D. chrysippus* specimens, but it was no longer detectable in the body of 15 out of 21 specimens within the following 8 days (Fig. 2). In a similar study comparing the sequestration of the two cardenolides by *D. plexippus* and *D. gilippus*, it was shown that the latter species sequesters calactin only (Mebs et al., 2012). Whether selective transport mechanisms favouring the uptake of that cardenolide, or metabolic processes are involved, such as epimerization of the 3′-hydroxy group of calotropin to calactin, remains to be investigated. Polar compounds such as calotropin and calactin were found to be preferably sequestered by *Danaus* species (Nelson, 1993b), but this