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SEQUESTRATION, MAINTENANCE, AND TISSUE DISTRIBUTION OF PYRROLIZIDINE ALKALOID *N*-OXIDES IN LARVAE OF TWO *Oreina* SPECIES

ADELHEID EHMKE,[1] MARTINE RAHIER,[2] JACQUES M. PASTEELS,[3] CLAUDINE THEURING,[1] and THOMAS HARTMANN[1,*]

[1]*Institut für Pharmazeutische Biologie*
Technischen Universität Braunschweig
D-38106 Braunschweig, Germany

[2]*Écologie Animale*
Université de Neuchâtel
rue Emile Argand 11, CH-2007 Neuchâtel, Switzerland

[3]*Laboratoire de Biologie Animale et Cellulaire*
Université Libre de Bruxelles
B-1050 Bruxelles, Belgium

Abstract—*Oreina cacaliae* and *O. speciosissima* are leaf beetles that, as larvae and adults, sequester pyrrolizidine alkaloid *N*-oxides (PAs) as defensive compounds from their host plants *Adenostyles alliariae* and *Senecio nemorensis*. As in most *Oreina* species, *O. speciosissima* is also defended by autogenously produced cardenolides (mixed defensive strategy), whereas *O. cacaliae* does not synthesize cardenolides and is exclusively dependent on host-plant-acquired PAs (host-derived defense). Adults of the two *Oreina* species were found to have the same PA storage capacity. The larvae, however, differ; larvae of *O. speciosissima* possess a significantly lower capability to store PAs than *O. cacaliae*. The ability of *Oreina* larvae to sequester PAs was studied by using tracer techniques with ^{14}C-labeled senecionine *N*-oxide. Larvae of the two species efficiently take up [^{14}C]senecionine *N*-oxide from their food plants and store the alkaloid as *N*-oxide. In *O. cacaliae*, there is a slow but continuous loss of labeled senecionine *N*-oxide. This effect may reflect the equilibrium between continuous PA uptake and excretion, resulting in a time-dependent tracer dilution. No noticeable loss of labeled alkaloid is associated with molting. Senecionine *N*-oxide is detectable in all tissues. The hemolymph is, with ca. 50–60% of total PAs, the major storage compartment, followed by the integument, with ca 30%. The alkaloid concentration in the hemolymph is approximately sixfold higher than in the solid tissues. The selectivity of PA sequestration in larvae is comparable to PA sequestration in the bodies of adult beetles.

Key Words—*Oreina* spp., Coleoptera, Chrysomelidae, alkaloid sequestration, pyrrolizidine alkaloid *N*-oxide, senecionine *N*-oxide, chemical defense, larval defense.

*To whom correspondence should be addressed.

INTRODUCTION

Adult leaf beetles of the Alpine genus *Oreina* are chemically protected by defensive secretions released from exocrine glands located in the elytra and pronotum (Pasteels et al., 1988a, 1994). Chemical defense in *Oreina* is primarily autogenous by de novo synthesized cardenolides (Pasteels et al., 1996; Dobler et al., 1996). Only a few *Oreina* species feeding on plants belonging to the Asteraceae, tribe Senecioneae, sequester pyrrolizidine alkaloid *N*-oxides (PAs) from their host plants (Pateels et al., 1996). Since the first report (Pasteels et al., 1988b), the mechanism of PA sequestration in adults of *Oreina* has been studied in detail (Pasteels et al., 1996; Hartmann et al., 1997). *O. cacaliae* is the only species that does not endogenously produce cardenolides, and instead sequesters PAs from its host plants, *Adenostyles alliariae* and *Senecio nemorensis*. In beetles, plant-acquired PAs are taken up into the hemolymph. Absorbed PAs are stored in the body, from where they are partly translocated into the secretory glands and released with the defensive secretions. Thus, in beetles, PAs are stored in two different compartments, the body (i.e. hemolymph and integument) and the glands (Rowell-Rahier et al., 1991; Ehmke et al., 1991; Pasteels et al., 1992, 1995; Hartmann et al., 1997).

Unlike adults, *Oreina* larvae do not have any mechanical protection and do not possess defensive glands. However, they are chemically defended. They autogenously produce cardenolides, which they store in their bodies (Eggenberger and Rowell-Rahier, 1993; Dobler and Rowell-Rahier, 1994). This is remarkable, since in adults of *Oreina* species, cardenolides have not been detected in any tissue except the glands and, in oviparous species, the eggs (Dobler and Rowell-Rahier, 1994; Pasteels et al., 1996). Species that sequester PAs from their host plants as adults, such as *Oreina cacaliae*, *O. elongata*, *O. intricata*, and *O. speciosissima* (Pasteels et al., 1995, 1996; Hartmann et al., 1997), also sequester PAs as larvae (Dobler and Rowell-Rahier, 1994).

In this study, we analyzed the capability and capacity of larvae and adults of *O. cacaliae* and *O. speciosissima* to sequester PAs from their two PA-containing food plants, *A. alliariae* and *S. nemorensis*. Subsequently, tracer feeding experiments with radioactively labeled senecionine *N*-oxide were performed to study alkaloid uptake, maintenance, and tissue distribution in larvae. The tracer techniques had been successfully applied in the past to study PA uptake and metabolism in *Oreina* adults (Ehmke et al., 1991; Pasteels et al., 1992).

METHODS AND MATERIALS

Insects. For chemical analysis, adults of *O. cacaliae* (Schrank) and *O. speciosissima* (Scopoli) were collected at Tschiertschen (Graubünden, Switzerland) at elevations of 1800–2000 m (*O. cacaliae*) and 1500–1800 m (*O. speciosissima*) feeding on *Adenostyles alliariae* (Gouan) Kern. and *Senecio nemorensis* L. (s. str.) (Asteraceae, Senecioneae). In the laboratory, the beetles were kept on their food plants at room temperature or in a cool-chamber at 8°C until use. The offspring were raised on *A. alliariae* and *S. nemorensis.*

For tracer experiments, *O. cacaliae* adult females were collected in Appenzell (Switzerland) at elevation of 1300 m on *A. allariae* and *O. speciosissima* adult females in Zastler (Black Forest, Germany, 1170 m) on *Petasites paradoxus*, the preferred larval food plants in the field. *P. paradoxus* does not contain PAs in its leaves. Larvae produced by these females were kept on their respective food plants at 17°C until they reached the larval stage required in the experiment.

Tracer Feeding Experiments. [^{14}C]Senecionine *N*-oxide (1.07 GBq/mmol) was prepared biosynthetically from [1,4-^{14}C]putrescine (4.4 GBq/mmol, Amersham Buchler, Braunschweig, Germany) by using root cultures of *Senecio vulgaris* and was subsequently purified (Hartmann, 1994). [^{14}C]Senecionine *N*-oxide prepared by this method was chemically and radiochemically pure. For the feeding experiments, 10 μl of a methanolic solution of the tracer (ca. 2 kBq corresponding to ca. 3000 cps) was painted on the surface of leaf disks (6–10 mm diameter depending on the larval age) prepared from fresh leaves of *A. alliariae* (*O. cacaliae*) and *P. paradoxus* (*O. speciosissima*). After evaporation of the solvent, the disks were placed in Petri dishes (5 cm diameter) with one larva (last instar) each. Larvae were allowed to feed 24 hr. After 24 hr, larvae that had eaten less than half of the leaf offered were not considered. The remaining individuals were transferred to fresh untreated leaves and allowed to feed until termination of the experiment.

Individual larvae were extracted twice with 2 ml methanol. After centrifugation, total radioactivity was determined by scintillation counting (Rialuma, Baker). The remains of the tracer leaves were collected and analyzed. Total radioactivity evaluated for larval extracts was related to the amount of "ingested tracer." Ingested tracer (100%) is defined as total radioactivity offered minus total radioactivity recovered from the remains of the tracer-treated food leaf.

Separation of the labeled extracts to localize senecionine *N*-oxide and any of its metabolites was achieved by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) according to Ehmke et al. (1991) and Hartmann and Dierich (1998). Radioactively labeled compounds were located by means of a TLC multichannel analyzer (Rita-32a, Raytest) and a HPLC radioactivity monitor LB-506D (Berthold).

Dissection. The integument of third- and fourth-instar larvae was dorsally opened with small scissors and the hemolymph was collected in glass capillary tubes and stored in methanol until analysis. Subsequently, the gut and the fat body were prepared and immediately preserved in methanol. The residual tissue was then called integument.

Gas Liquid Chromatography (GLC). The true PA pattern of the food plant leaves as well as that of the *Oreina* larvae and adults was qualitatively and quantitatively evaluated as described by Witte et al. (1993) and Pasteels et al. (1995). The identity of individual alkaloids was confirmed by combined gas chromatography–mass spectrometry (GC-MS) (Witte et al., 1993; Hartmann et al., 1997).

RESULTS

PA Contents and Patterns in Larvae and Adults. Field-collected adults of *O. cacaliae* and *O. speciosissima* and their offspring were raised on their two food plants *Senecio nemorensis* and *Adenostyles alliariae* in the laboratory and were then analyzed for their total PA contents and concentrations (Table 1). The amount and concentration of PAs sequestered by either larvae (analyzed after the last larval molt) or adults of the two species is not affected by the food-plant species, although the two species contain structurally different PAs (Figure 1) (Hartmann et al., 1997). On the two food plants, larvae but not adults of *O.*

TABLE 1. AMOUNTS AND CONCENTRATIONS OF TOTAL PAs SEQUESTERED BY LARVAE AND ADULTS OF *Oreina cacaliae* AND *O. speciosissima* ON TWO HOST PLANTS[a]

			Total PAs (μg ± SD)	
	N	Host plant	per individual	per gram/g fresh weight
Oreina cacaliae				
Larvae	19	*S. nemorensis*	77.7 ± 31.7[1]	2031 ± 891[2]
Adults	9	*S. nemorensis*	74.6 ± 12.6	909 ± 101
Larvae	19	*A. alliariae*	68.8 ± 25.8[3]	1700 ± 587[4]
Adults	9	*A. alliariea*	98.0 ± 6.1	1187 ± 96
Oreina speciosissima				
Larvae	16	*S. nemorensis*	12.2 ± 6.3[1]	428 ± 169[2]
Adults	4	*S. nemorensis*	51.5 ± 16.3	797 ± 76
Larvae	13	*A. alliariae*	13.0 ± 6.6[3]	339 ± 167[4]
Adults	2	*A. alliariae*	94.0	1465

[a]The PA contents and concentrations of *O. cacaliae* and *O. speciosissima* feeding on the respective host plants are significantly different; Student's *t* test, [1,2]$P < 0.005$; [3,4]$P < 0.01$.

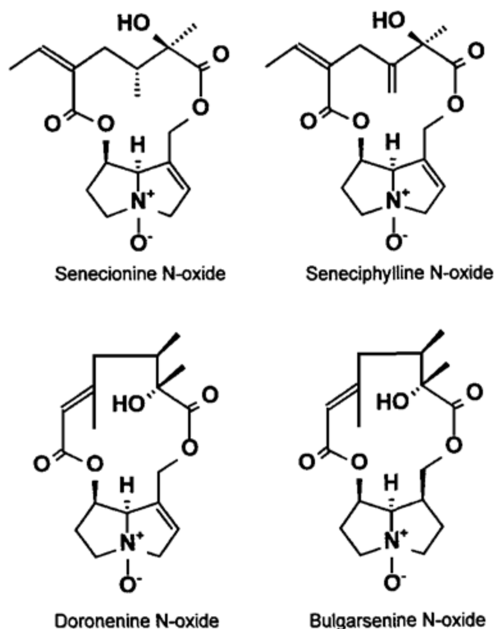

FIG. 1. Major PAs sequestered by *Oreina* larvae from their host plants *Adenostyles alliariae* (*N*-oxides of seneciphylline and senecionine) and *Senecio nemorensis* (*N*-oxides of doronenine and bulgarsenine).

cacaliae showed a higher capacity (Student's *t* test, $P < 0.005$, *S. nemorensis*; and $P < 0.01$, *A. alliariae*) to sequester PAs than larvae of *O. speciosissima* (Table 1).

Seneciphylline *N*-oxide is the major PA found in larvae and adults feeding on *A. alliariae*, and it is occasionally accompanied by trace amounts of senecionine *N*-oxide. *Oreina* larvae and adults feeding on *S. nemorensis* contain doronenine *N*-oxide (65–85%) and bulgarsenine *N*-oxide (15–35%) as major alkaloids (data not shown).

Sequestration of [^{14}C]Senecionine N-*Oxide.* Larvae (fourth instar) of the two *Oreina* species were pulse-fed with [^{14}C]senecionine *N*-oxide for 24 hr and then transferred to untreated host plant leaves. The percentage of ingested radioactivity absorbed was assayed 24–96 hr (*O. cacaliea*) and 24–72 hr (*O. speciosissima*) following termination of the tracer pulse-feeding. Larvae of the two species efficiently sequester the labeled senecionine *N*-oxide. Approximately 20–30% of the ingested alkaloid is absorbed into the body (Figure 2). In *O. cacaliae*, the sequestered labeled alkaloid *N*-oxide is lost over time, whereas it remains stable in *O. speciosissima*. A two-factor ANOVA with radioactivity

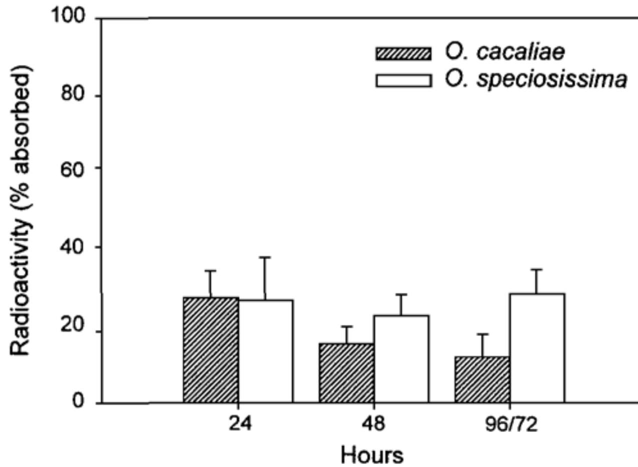

FIG. 2. Sequestration of [^{14}C]senecionine *N*-oxide by larvae (fourth instar) of *O. cacaliae* and *O. speciosissima*. Total ingested radioactivity was set 100%. Number of individuals analyzed at the time indicated, *N* 11–12, mean % ± SD. The tracer (3000 cps) was applied on leaf disks and offered to single larvae. After 24 hr, larvae were removed and allowed to continue feeding on untreated leaves for another 24, 48, and 96 (*O. cacaliae*) or 72 hr (*O. speciosissima*) as indicated.

recovered as dependent variable, revealed an effect of the factor time ($F_{2,61}$ = 8.259; $P = 0.001$), of the factor species ($F_{1,61}$ = 21.384, $P < 0.001$) as well as an interaction ($F_{2,61}$ = 8.409, $P = 0.001$). The mean radioactivity recovered from *O. speciosissima* larvae (26.16, SD = 7.97, $N = 33$) is higher than that of *O. cacaliae* larvae (18.73 ± 8.79, $N = 34$).

TLC and HPLC analysis of larval methanol extracts revealed that most of the soluble radioactivity was recovered as senecionine *N*-oxide (>85%). Only a small proportion of the sequestered radioactivity was recovered as a polar metabolite (Hartmann et al., 1999). Labeled senecionine was either completely absent or detectable in traces only.

In a long-term experiment, young (i.e. one day after molting) second and third instars of *O. cacaliae* were fed [^{14}C]senecionine *N*-oxide. After 24 hr, larvae were transferred to untreated host-plant leaves and analyzed after time intervals as indicated in Figure 3. The two instars transfer the sequestered alkaloid to the next instar. However, larvae apparently lose sequestered [^{14}C]senecionine *N*-oxide during their development. At the end of the fourth instar, larvae had lost by excretion more than 80% of the alkaloid *N*-oxide sequestered at the beginning of the third instar (Figure 3). No obvious loss of alkaloids could be observed during molting. In a two-factor ANOVA with recovered radioactivity as de-

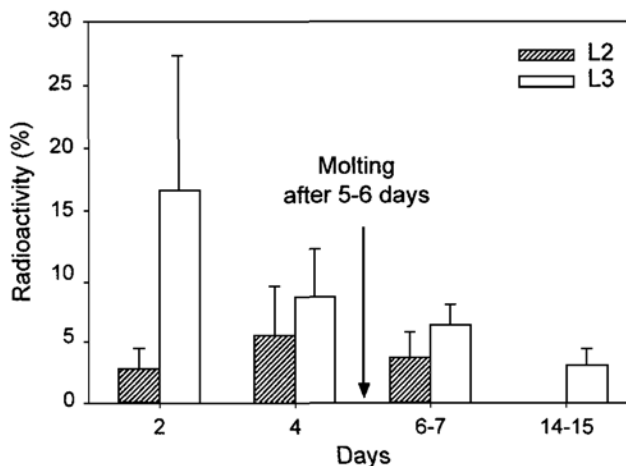

FIG. 3. Retention and loss of ingested [^{14}C]senecionine *N*-oxide during growth of *O. cacaliae* larvae. Larvae at the beginning of the second (L2) and third (L3) instars were pulse fed with labeled alkaloid (3000 cps each). After 24 hr, larvae were removed and allowed to continue feeding on untreated leaves. Ingested radioactivity was set 100%. Number of samples analyzed at each time: N = 4–6; mean % ± SD.

pendent variable, there is a significant effect of the factor larval instar ($F_{1,25}$ = 15.83, P = 0.001), but not of the factor time ($F_{2,25}$ = 2.136, P = 0.14) up to days 6–7. The interaction was not significant ($F_{2,25}$ = 2.92, P = 0.07). On average, third instars (10.48 ± 6.68, N = 18) retain more radioactivity than second instars (4.19 ± 2.78, N = 18). In third instars, the decrease of radioactivity with time becomes significant if amounts at days 14–15 are included in the analysis ($F_{3,15}$ = 4.3900, P = 0.0209).

Distribution of Sequestered [^{14}C]Senecionine N-*Oxide Between Larval Tissues.* Radioactively labeled alkaloid *N*-oxide was detected in all major larval tissues (Figure 4). Almost 50–60% of total senecionine *N*-oxide was found in the hemolymph, followed by the integument (27–31%). Fat body and gut contained less than 8% each. Only traces of radioactivity (<3%) were associated with the exuvia; this corresponds well with the observation that no loss of radioactivity was observed during molting (Figure 3). A rough calculation of the tissue concentrations revealed almost the same concentrations in the solid tissues and an approximately sixfold higher concentration in the hemolymph, the major PA storage compartment. The solid tissues are necessarily contaminated with hemolymph; therefore, the total amount and concentrations measured for these tissues are overestimated. This stresses the importance of the hemolymph as a major storage compartment.

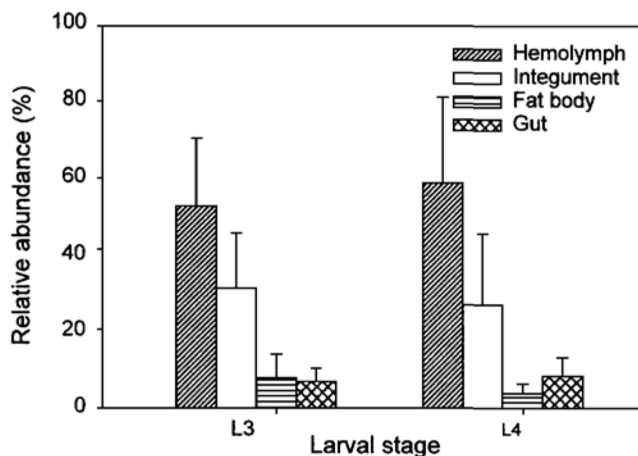

FIG. 4. Distribution of radioactivity associated with hemolymph and different solid tissues of *O. cacaliae* larvae. L3 and L4 larvae were individually fed with [^{14}C]senecionine *N*-oxide (3000 cps each). After 24 hr, larvae were removed and allowed to continue feeding for another 24 hr and were then dissected. Mean % ± SD (*N* = 4 in each series).

DISCUSSION

The amount and concentrations of PAs sequestered in the bodies of larvae and adults of *O. cacaliae* and *O. speciosissima* are of the same order of magnitude as reported recently by Rowell-Rahier et al. (1991), Dobler and Rowell-Rahier (1994), and Pasteels et al. (1996). In comparison to *O. cacaliae*, the PA storage capacity of *O. speciosissima* is less efficient, especially in the larval stage. This may reflect different defensive strategies of the two species: host-derived defense in *O. cacaliae* and mixed defensive strategy in *O. speciosissima* (Dobler and Rowell-Rahier, 1994; Pasteels et al., 1995, 1996).

No significant qualitative differences in the alkaloid patterns were observed between larval and adult populations feeding on the two host plants *A. alliariae* and *S. nemorensis*. Larvae and adults feeding on *A. alliariae* preferentially sequester seneciphylline *N*-oxide, the major plant PA. Larvae and adults feeding on *S. nemorensis* sequester doronenine *N*-oxide, accompanied by a smaller proportion of its 1–2 saturated derivative bulgarsenine *N*-oxide (Figure 1). The preferential sequestration of the 1–2 unsaturated doronenine *N*-oxide is remarkable because this alkaloid is a minor component in *S. nemorensis*, which contains bulgarsenine *N*-oxide as the major alkaloid (Pasteels et al., 1996; Hartmann et al., 1997).

PA sequestration in larvae is efficient and specific. Radioactively labeled

senecionine *N*-oxide is sequestered as *N*-oxide and is almost completely retained in the body. Only a small proportion of the *N*-oxide is transformed into a polar metabolite that recently was identified as a senecionine *O*-glycoside (Hartmann et al., 1999). The sequestered alkaloid *N*-oxide is retained during molting. There is, however, a continuous slow loss during larval growth (Figure 3). This loss was also observed in the short-term experiment illustrated in Figure 2; it involves *O. cacaliae*, not *O. speciosissima*. This seems to contradict the fact that *O. cacaliae* is the species with the more advanced sequestration strategy and a higher PA storage capacity. Here, we have to take into consideration that we are discussing the results of pulse-feeding tracer experiments. *O. cacaliae* larvae feeding on their PA-containing food plant *A. alliariae* have a high systemic load of PAs. This load, defined by the PA storage capacity of the larvae, should be characterized by a well-tuned balance between PA absorption and excretion. The intake of new PAs should be balanced by continuous PA excretion. This, in turn, would result in a continuous dilution of the population of labeled PAs sequestered during the feeding pulse. With *O. speciosissima*, we have a different situation: The larvae fed on their preferred food plant *P. paradoxus*, which does not contain PAs in its leaves, and consequently they are devoid of PAs. During the feeding pulse with labeled senecionine *N*-oxide, larvae only ingest the labeled PAs, which represent a small absolute amount, i.e., <0.5 μg senecionine *N*-oxide per larva. Most likely the PA threshold concentration in the hemolymph will not be reached. As a consequence, the population of sequestered labeled senecionine *N*-oxide remains trapped in the larva's body. Although this interpretation appears to be reasonable, further kinetic studies are needed to confirm it.

The preferential storage of the labeled senecionine *N*-oxide in the hemolymph and a low but almost equal concentration in the solid organs of larvae indicate the absence of specific storage compartments. Here, larvae behave like adult beetles (Pasteels et al., 1992; Hartmann et al., 1997). A similar nonspecific tissue distribution of sequestered compounds, which appears to depend on the chemical polarity of the constituent, has been reported for the tissue distribution of plant-acquired cardenolides in the monarch butterfly (Brower et al., 1988). Adults and larvae of PA-sequestering *Oreina* species are able to sequester PA *N*-oxides from their host plants in a relatively nonspecific manner. Adults transfer PA *N*-oxides from the hemolymph into the exogenous glands where the alkaloid *N*-oxides reach concentrations up to 0.3 mol/liter (Rowell-Rahier et al., 1991; Hartmann et al., 1997), which is 50- to 100-fold higher than the concentrations calculated on the fresh-weight basis for larvae and adults in this study (see Table 1). Phylogenetic studies indicate that *O. cacaliae* switched from autogenous defense by cardenolides to PA sequestration (Dobler et al., 1996; Pasteels et al., 1996; Hsiao and Pasteels, 1999). One may speculate that a switch from autogenous defense to host-plant-acquired PA *N*-oxides occurred in two steps. First, larvae and adults attained the ability to sequester PA *N*-oxides and store

them in their bodies. Second, adult beetles attained the ability to transfer PA *N*-oxides from the hemolymph into the gland cells, where they are concentrated and excreted with the defensive secretion. In comparison to body sequestration, gland sequestration is a more specific and selective process. A number of PA *N*-oxides that are found in the bodies of larvae and adults are never transferred into the glands. For instance, adults of *O. cacaliae* that feed on *S. nemorensis* sequester in their bodies the *N*-oxides of bulgarsenine and doronenine, but transfer only the potentially toxic doronenine into the glands (Pasteels et al., 1996; Hartmann et al., 1997). To obtain more support for the idea of a two-step adaptation to host-derived defense in leaf beetles it would be interesting to see whether there are *Oreina* species sequestering PAs in their bodies but still incapable of transferring them into the secretions.

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