

**Chemical defense in chrysomelid leaf beetles:
Storage of host derived pyrrolizidine alkaloids versus de novo synthesized
cardenolides.**

Jacques M. Pasteels, Adelheid Ehmke*, Florian Eggenberger**, Martine Rowell-Rahier**
& Thomas Hartmann*

Laboratoire de Biologie Animale et Cellulaire, Faculté des Sciences, ULB, 50 av. F.D.
Roosevelt, Brussels, B-1050 Belgium.

* Pharmazeutische Biologie, Technische Universität Braunschweig, Mendelssohnstrasse 1,
Braunschweig, d-3300 Germany.

** Zoologisches Institut, Rheinsprung 9, Basel, CH-4051 Switzerland.

Exocrine chemical defense is widespread in the Chrysomelinae [1]. We have recently demonstrated the sequestration of N-oxides of pyrrolizidine alkaloids (PA N-oxides) in specialised exocrine glands as well as in the body of the leaf beetles *Oreina cacaliae* (Chrysomelinae) [2, 3]. There thus seems to be two compartments for the storage of PA N-oxides in this species: first, large quantities are stored at low concentration in the body; and secondly, small quantities are stored at high concentration in the defensive glands. These results suggested that the PA stored in the body can be translocated into the secretion. Here to test this hypothesis, we examine the fate of ingested ^{14}C labelled PA N-oxide during a period of 25 days, paying particular attention to the replacement of the secretion after it has been experimentally removed.

The storage of PA N-oxides for defensive purpose in *O. cacaliae* contrasts with the defensive strategy of other *Oreina* species which produce complex mixtures of cardenolides not present in their food-plant. Here we select *O. gloriosa* as a typical cardenolide producer. That cardenolides are biosynthesised de novo from ubiquitous phytosterols was demonstrated clearly in the related genus *Chrysolina* [4]. In *Chrysolina*, the cardenolides (site of biosynthesis still unknown) are found mostly, if not exclusively, in the defensive glands and not in the body of the beetle (D. Dalozé, personal communication). If this was also the case in the *Oreina* producing cardenolides, the chemical defense of the different species would not only vary in the type of compound used and in their origin, but also in their tissue distribution in the beetles. In the species sequestering plant compounds, the ability to translocate compounds present in the body directly into the glands when they are empty might be advantageous (time and cost wise) over refilling of the glands with newly synthesized compounds.

O. cacaliae feeding on *Adenostyles alliariae* were collected in May 1991 in "Le Howald" (Alsace, France). The defensive secretion was removed and the beetles were starved for 24 hr prior to the start of the experiment during which they were fed with *Adenostyles alliariae*. On day 1, 40 beetles were each given 2,000 cps of ^{14}C senecionine N-oxide dissolved in 10ul of methanol and applied to a leaf disc. After 24hr, the beetles which had not eaten at least half of the disc were discarded. Six beetles were kept undisturbed until the end

of the experiment on day 25 when their secretion was removed for the first time, after which the beetles were killed. The remaining beetles were divided into 3 groups. The secretion of all the beetles was removed on day 8 and the beetles of the first group (n=7) were then killed, on day 15 the remaining beetles were milked a second time and then the second group of beetles (n=6) were killed, finally on day 25 the third group of beetles (n=7) were milked a third time and then killed. The beetles were killed by freezing after weighing. The volume of the individual secretions were measured. Thus we have groups of beetles which need to renew their secretion respectively once (group 1 on day 8 and undisturbed group on day 25), twice (group 2 on day 15) and three times (group 3 on day 25). The radioactivity was measured in the pooled secretions of each group and in the body of each individual beetle. PA in the body were extracted first twice in methanol and then the remains in lumasolve. To check for possible metabolism of the ingested radioactive senecionine N-oxide, TLC of the secretion and methanol extract of beetle body was performed.

The volume of the secretion (mean 115 nl, sd. 56, n=26) does not vary significantly between the different collection (Anova: $F=1.08$, ddf: 25, $p=.37$).

The radioactivity recovered (cumulative total and cps/ beetle or secretion) over a period of 25 days is shown in Fig 1. One week after the begin of the experiment (day 8), an average of 9% of the total radioactivity available was taken up and found in the beetles which is comparable (11%) to previously published data [2]. The total radioactivity recovered stays similar (8%) for at least the 25 days when the beetles are undisturbed. The quantity of radioactive senecionine N-oxide decreases in the body when the secretions are collected successively on day 8, 15 and 25 (Fig 1) and each of these secretion is labelled. The repeated presence of radioactivity in the secretion shows unambiguously that the beetles translocate stored compounds into the secretion after its depletion. These compounds are probably stored in the body in a quantity sufficient to refill the glands twice. Because the secretion of the beetles from each group were pooled for the measurement of radioactivity, no statistical analysis of the secretion radioactivity is possible.

The concentration of radioactive senecionine N-oxide in the secretion decreases from one collection to the next (see Table 1). This is expected if the stored labelled PA remaining

in the body after each milking is diluted by unlabelled plant PA ingested during feeding. However, it is interesting that the concentration of labelled N-oxide in the secretion remains high compared to that in the body: in the secretion it reaches 373 cps/ul while in the body only 2.1 cps/mg are found (i.e. a difference of more than two orders of magnitude). Storage in defensive glands isolated from the rest of the body seems to favor higher concentration which must be more active in defense. TLC of the secretions shows no metabolism of the senecionine N-oxide ingested.

O. gloriosa were collected in July 1991 in Saas Grund (Swiss Alps) on their foodplant *Peucedanum ostruthium* (Apiaceae). The hemolymph of 5 randomly selected beetles, which were killed by freezing, was taken up in capillary tube. The hemolymph sample was vortexed in 100 μ l MeOH and centrifuged. The supernatant was evaporated and redissolved in 25 μ l MeOH. Pronotal and elytral secretions of 22 beetles were removed with capillary tubes, dissolved in 200 μ l MeOH, evaporated to dryness and redissolved in 25 μ l MeOH for TLC. The beetles were killed by freezing and dipped in MeOH to remove secretion contamination of the surface of the cuticle. Each beetle was dissected to separate the head with the gut, the elytra, the wings, the abdomen, the prothorax and the meso-/metathorax. For the 22 beetles the respective body parts were pooled, homogenized in 3 ml MeOH and centrifuged. The supernatants were evaporated and the residues redissolved in 25 μ l MeOH. Cardenolide analysis was performed using TLC. The samples were spotted on silica gel 60 plates (Merck) and developed in $\text{CHCl}_3/\text{CH}_3\text{OH}$ 8:2. The plates were air dried and visualized by spraying with Kedde reagent [refs. in 5].

The secretion sample afforded 12 purple spots, typical of cardenolides. However, neither the hemolymph sample nor the samples of the dissected body parts showed cardenolide-positive spots although this method allows to visualize cardenolide spots of 2 μ g. Thus, cardenolides of adult *O. gloriosa* are detected only in the defensive secretions although they are found in extracts of the larvae of this species, which do not have the defensive glands found in the adults. Contrary to *O. cacaliae* sequestering PAs from plant, there seems to be no long term storage of toxins outside the defensive glands in species which synthesize their own cardenolides (e.g. *O. gloriosa*).

Intuitively, the sequestration of plant toxins compared to the synthesis of autogenous compounds has advantages, but also a potential drawback. Sequestration could be metabolically less costly than *de novo* synthesis, especially if the insect has organs such as defensive glands in which toxins can be stored at high concentration. On the other hand, sequestration could provide the herbivore with a less reliable defense than *de novo* synthesis because of the intrinsic plant heterogeneity. Indeed, *O. cacaliae* feeds in the field on *Adenostyles alliariae* and *Senecio fuchsii*, but this beetle is only able to sequester in its defensive glands the compounds typical of the first plant species and not those of the second one. Interestingly storage in the body is less specific [2]. Even more unexpected is the fact that *O. cacaliae* also feeds on *Petasites paradoxus* in the field since this plant does not contain PAs in its leaves [3]. Moreover within one field site, different plants of *Adenostyles alliariae* have been shown to be highly variable (from 0 to 10 000 ppm PA in the dry leaves) [6]. The ability to store PAs in the body over a long period of time and to translocate them into the glands when these are emptied, is clearly a way to buffer the beetle defensive characteristics and to counteract the phytochemical variability. It might even be necessary for sequestration of toxins to be an evolutionary stable defensive strategy in herbivores.

Legends

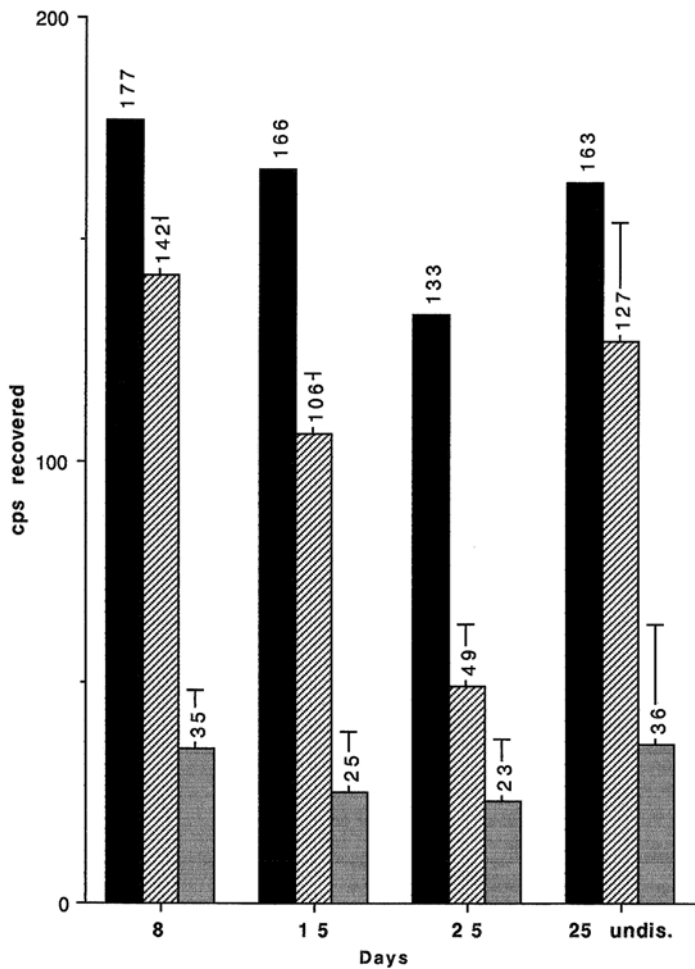
Fig. 1: Radioactive Senecionine N-oxide recovered at different time after feeding of *O. cacaliae*: given are the cumulative totals (= sum of content of the body and of the secretions removed to date), the contents per body (with standard error) and per secretion. The secretion is removed at the beginning of the experience and then either once (day 8 or 25 undis.), twice (day 15) or three times (day 25). The radioactivity of the bodies is significantly different between day 8 and 25 (Anova $F= 5.55$, $ddf= 25$, $p= 0.005$ and Scheffe F test = 4.88) and between day 25 and 25 undisturbed (Scheffe F test = 3.16).

Table1: concentration of radioactive senecionine N-oxide in the body and the secretion of *O. cacaliae* when the secretion has to be renewed once (day 8 and 25 undis.), twice (day 15) or three times (day 25). The concentrations decreases significantly between the bodies (Anova $F=4.45$, $ddf= 25$, $p=0.013$) after the first and third secretions removal (Fischer PLSD= .807), after the second and third (Fischer PLSD= .84), or between the third secretion removal and the the undisturbed group (Fischer PLSD= .84).

Day	cps/ mg body	cps/ ul secretion
8	2.12 (sd.69)	370
15	1.68 (sd.68)	240
25	0.75 (sd.66)	160
25 undis.	1.81 (sd.87)	220

1. Pasteels, J.M., Rowell-Rahier, M., Braekman, J.C., Daloze, D., and Duffey, S.: *Experientia* 45, 295 (1989).
2. Ehmke, A., Rowell-Rahier, M., Pasteels, J.M. & Hartmann, T.: *Journal of Chemical Ecology* 17, 2367 (1991).
3. Rowell-Rahier, M., Witte, L., Ehmke, A., Hartmann, T. & Pasteels, J.M.: *Chemoecology*: 2, 41 (1991).
4. Van Oycke, S., Braekman, J.C., Daloze, D., and Pasteels, J. M.: *Experientia* 43, 460 (1987).
5. Daloze, D, and pasteels, J.M. : *Journal of Chemical Ecology* 5, 65 (1979)
6. Donati, F.: *Diplomarbeit Universität Basel* 1992.

Fig. 1



■ Cumulative total (body+secretions removed to date)
 ▨ Body
 ■ Secretion