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HOST-DERIVED PYRROLIZIDINE ALKALOIDS IN *OREINA* LEAF BEETLES: PHYSIOLOGICAL, ECOLOGICAL AND EVOLUTIONARY ASPECTS

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Abstract

Sequestration of pyrrolizidine alkaloids (PAs) from Asteraceae-Senecioneae is described in the adults and larvae of *Oreina cacaliae*, *O. spectiosissima*, *O. elongata* and *O. intricata*. The three last species also secrete autogenous cardenolides. *Oreina* spp. feeding on Apiaceae or exclusively on Astraceae-Cardueae only secrete autogenous cardenolides. Molecular phylogenies indicate that the production of autogenous cardenolides is ancestral. Pure sequestration in *O. cacaliae* evolved from a mixed defense (PA sequestration and cardenolide production). A reverse evolution to pure cardenolide production from the mixed defense seems possible. The advantages of the different defenses as well as the ecological constraints acting on their evolution are discussed. It is suggested that host-plant shifts during evolution, more than the selective pressure of natural enemies are responsible for the different modes of defense.

Introduction

Chemical defense is prominent in aposematic adult Chrysomelinae, the defensive substances being released from exocrine glands located in the elytra and pronotum (reviews in Pasteels *et al.*, 1988a, 1994). Although most chrysomelines are specialist herbivores, often feeding on plants containing secondary compounds known to be toxic and/or deterrent for both vertebrates and invertebrates, chemical defense in chrysomeline beetles appears to be primarily autogenous, and rarely and secondarily host-derived. So far, sequestration of plant compounds for beetle defense in the chrysomelines has been documented in only a few *Oreina* species feeding on Asteraceae, tribe *Senecioneae*, from which they accumulate pyrrolizidine alkaloids (hereafter abbreviated to PAs) in their defensive secretion and hemolymph.

Since its first report in *O. cacaliae* (Schrank) (Pasteels *et al.* 1988b), sequestration of PAs has been the object of several investigations in our laboratories and the purpose of this review is both to summarize the already published results, and to incorporate some unpublished data. The emphasis will be not only on mechanistic aspects (for example, specificity of sequestration, metabolism, storage compartments, turnover), but also on ecological (defensive efficiency, cost, influence of local host-plant community) and evolutionary aspects.

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The occurrence of sequestration of pyrrolizidine alkaloids in the genus *Oreina*

Chemical analyses of the defensive secretions of *Oreina* spp. have revealed three different patterns (Table 1, Pasteels *et al.*, 1995).

Nine species feeding on either Apiaceae or Asteraceae, tribe Cardueae, secrete cardenolides. Species not listed in Table 1 are *O. gloriosa* (Fabricius), *O. bifrons* (Fabricius), *O. variabilis* (Weise) and *O. speciosa* (Linné). Cardenolides are frequently secreted in related taxa (*Ambrostoma* and *Chrysolina* spp.) among the Chrysolinina (Pasteels *et al.*, 1994) and in some Doryphorina (*Zygogramma* and *Calligrapha*, Timmermans *et al.*, 1992). Cardenolides were never reported in the host-plants of these

Table 1. Distribution of de novo synthesized cardenolides and sequestered PAs in the defensive secretions of *Oreina* spp.

Species	Defensive compounds	Host-plants	Localities
O. viridis	cardenolides	*Meum athamanticum* (Apiaceae)	Tschiertschen
O. frigida	cardenolides	*Meum athamanticum* (Apiaceae)	Hörnlihütte
O. bidentata	cardenolides	*Leuzea rhapontica* (Asteraceae-Cardueae)	Bosco Gurin
O. coerulea	cardenolides	*Centaurea nemoralis* (Asteraceae-Cardueae)	Calmbach
O. virgulata	cardenolides	*Circium spinosissimum* (Asteraceae-Cardueae)	Hörnlihütte
O. cacaliae	PAs	*Adenostyles alliariae*, *Senecio nemorensis* (Asteraceae-Senecioneae)	Tschiertschen
O. intricata	cardenolides + PAs	*Adenostyles alliariae* *Senecio nemorensis*, (Asteraceae-Senecioneae)	Tschiertschen
O. speciosissima	cardenolides + PAs	*Petasites albus*, *Senecio nemorensis* (Asteraceae-Senecioneae)	Tschiertschen
	PAs	*Adenostyles alliariae* (Asteraceae-Senecioneae)	Zinal
	cardenolides	*Petasites albus* (Asteraceae-Senecioneae)	La Lécherette
O. elongata	cardenolides + PAs	*Adenostyles leucophylla*, *Cirsium spinosissimum* (Asteraceae-Senecioneae, Cardueae)	Vallée des Merveilles
	PAs	*Adenostyles alliariae*, *Adenostyles alpina* (Asteraceae-Senecioneae)	Col du Lautaret
	cardenolides	*Circium spinosissimum* (Asteraceae-Cardueae)	Mattmark Dam

All localities are in Switzerland except for Calmbach (Germany), Vallée des Merveilles and Col du Lautaret (both France).
Modified from Pasteels *et al.* (1995).

beetles and are most probably autogenous. In *Chrysolina coerulans* (Scriba), living on mint, neosynthesis of cardenolides from cholesterol was proved by feeding the beetle with labelled cholesterol (Van Oycke *et al.*, 1987).

When leaf discs painted with [^{14}C]senecionine N-oxide were offered to *O. bifrons*, only one beetle out of five fed on them, demonstrating the deterrent activity of this PA on a non-adapted species (Rowell-Rahier *et al.*, 1991). The beetle which fed, as well as other beetles injected with [^{14}C]senecionine N-oxide, quickly excreted the compound (Ehmke *et al.*, 1991).

One species, *O. cacaliae*, only sequesters plant PAs, whereas three other species, *O. intricata* (Germar), *O. speciocissima* (Scopoli) and *O. elongata* (Suffrian), have a mixed defensive pattern, being able both to secrete autogenous cardenolides and to sequester plant PAs. Interestingly, some populations of two of these species, *O. speciocissima* and *O. elongata* were found, which rely on only one or the other chemical defense (see below). *O. frigida* accepts in the laboratory *Adenostyles alliariae* and is able to sequester PAs, but few informations are available on this species (Dobler *et al.*, in revision).

Sequestration in *Oreina cacaliae*

Adults

Sequestered PAs are stored in two different compartments: in the secretion and in the body (Rowell-Rahier *et al.*, 1991). Although the amount of PAs in one secretion (that is, in the quantity of secretion released from the glands on disturbance, circa 10 nmol, or 3.5 µg) is only about one fifth of the amount stored in the body (circa 50 nmol), the concentration of PAs in the secretions reaches 0.1 to 0.3 mol/l (Rowell-Rahier *et al.*, 1991). Since natural enemies first contact the secretion, high concentrations of PAs in this fluid could be relevant to deterring foes. In the body, the primary storage is in the hemolymph. Storage in tissues (for example, fat body) remains uncertain due to experimental difficulties. It was impossible to obtain tissue un-contaminated with hemolymph (without leakage of the PAs stored as polar N-oxides).

As in other insects (review in Malcolm, 1991), sequestration in *O. cacaliae* is a selective process, and more so in the secretion than in the body. With the exception of O-acetylseneciphylline, PAs related to the PA pattern found in *Adenostyles alliariae* were observed in the secretion of *O. cacaliae* collected on this plant (principally the N-oxides of senecionine and seneciphylline) (Table 2). These alkaloids were twelve-membered macrocyclic diester derivatives (senecionine group A_1, according to the classification of Hartmann and Witte, 1995). The proportion of PAs in the secretion does not completely mirror that observed in the plant. For example, the ratio senecionine/seneciphylline is much higher in the secretion than in the leaves of the food plant. Trace amounts of PAs (including retronecine) not detected in the leaves were sometimes observed in the secretion, suggesting some degradation of plant PAs by hydrogenation, loss of water and hydrolysis of the less stable allylic O^9-ester bond of seneciphylline/spartioidine (Rowell-Rahier *et al.*, 1991). However, no degradation of senecionine was observed when labelled senecionine was fed to the beetles (Ehmke *et al.*, 1991), and the traces of odd PAs detected in some secretions could be (at least in part) artefacts occurring during analysis.

Selective sequestration in the secretion is even better documented in beetles collected on other host-plants or fed with non-host plant PAs. *O. cacaliae* collected on

Table 2. Relative abundances of PAs (%) in *O. cacaliae* and its host plants

Alkaloids*	A.A.	S	B	S.F.	S	B	S.N.	S	B
Senecionine	1	14	6	–	50	27	–	tr	tr
Seneciphylline	81	45	86	–	–	tr	tr	31.6	22.9
Doronenine	–	–	–	–	–	–	tr	68.4	61.5
Spartioidine + platyphylline	16	16	5	–	50	16	–	–	tr
Bulgarsenine	–	–	–	–	–	–	92.4	tr	15.6
Neoplatyphylline	1	<1	<1	–	–	–	6.9	–	tr
Other PAs	1	25	8	–	–	7	0.6	–	–
Triangularine** groupe (B_1)	–	–	–	100	–	49	–		–

A.A.: *Adenostyles alliariae*, S.F.: *Senecio fuchsii*, S.N.: *Senecio nemorensis*.
S: secretion, B: body
*: Exclusively present as N-oxides.
**: Following the classification of Hartmann and Witte (1995). More than 7 different compounds, listed in Rowell-Rahier *et al.*, 1991.
The beetles were collected on *A. alliariae* in Appenzell (Zwitzerland), on *S. fuchsii* in Wasserliesen (France), and on *S. nemorensis* in Tschiertschen (Switzerland). The plants are from the same sites.
Modified from Rowell-Rahier *et al.*, 1991 and Pasteels *et al.*, 1995.

and fed with *Senecio nemorensis* contained in their secretion doronenine as the major constituent, but only traces of bulgarsenine. The reverse proportion was found in the leaves of *S. nemorensis* (Pasteels *et al.*, 1995). Doronenine and bulgarsenine are thirteen-mem-bered macrocyclic diesters (nemorensine group A_3 of Hartmann and Witte, 1995). Even more striking is the observation that beetles collected on or fed with *Senecio fuchsii* did not sequester in their secretion the PAs characteristic of this plant. These alkaloids are PA-monoesters belonging to the triangularine group B_1 of Hartmann and Witte (1995).

Monocrotaline N-oxide (an eleven-membered macrocyclic diester derivative, monocrotaline type D of Hartmann and Witte, 1995), never encountered in nature by the beetles, was not sequestered in the secretion when offered to the beetles (Rowell-Rahier *et al.*, 1991).

In summary, the plant species on which the beetles feed strongly influences the amount and kind of PAs sequestered. This is due to selective sequestration by the beetles: only some of the PAs belonging to the senecionine type of Hartmann and Witte (1995) are sequestered and not all with the same efficiency. To what extent the specific composition of the secretion of PAs is due to selective sequestration as opposed to metabolism of plant compounds remains to be discovered. An analysis of PA sequestration and metabolism in relation to structures will be published elsewhere (Hartmann *et al.*, in prep.). The beetles are sometimes found in nature on plants which were reported to produce PAs (Hartmann and Witte, 1995), but which were devoid of PAs in their leaves (for example, *Petasites paradoxus* and *P. albus*).

By contrast, sequestration is less specific in the body than in the secretion. For example, monocrotaline N-oxide fed to the beetle is sequestered in the body, as well as PAs characteristic of *S. fuchsii*. Bulgarsenine from *S. nemorensis*, only present as traces in the secretion, is present in significant amount in the body (Rowell-Rahier *et al.*, 1991; Pasteels *et al.*, 1995). Clearly the gut barrier is less selective to PAs than the gland barrier.

PAs are sequestered in secretion and body as N-oxides and not as tertiary com-

pounds. Experiments with labelled senecionine demonstrated that only the N-oxide is sequestered when fed to the beetle, but not the tertiary form (Ehmke *et al.*, 1991). This is an unexpected result, suggesting that sequestration is not simply a passive diffusion of non-polar compounds through the gut membrane. In this respect, beetles contrast strongly with moths and grasshoppers. In various arctiids and in the grasshopper *Zonocerus*, sequestration of PA N-oxides needs first a reduction in the gut of the N-oxide to the tertiary form, followed by a second oxidation in the body (Hartmann, 1996). Contrary to a previous claim (Wink and Schneider, 1988), sequestration in these insects is based on a passive diffusion of the reduced lipophilic tertiary alkaloid through the gut wall and does not require a specific carrier.

Retention of PAs in the body of *O. cacaliae* is long lasting. Radioactive senecionine N-oxide stored in the body is kept with little or no degradation or excretion for up to 25 days. This long-term storage of PAs in the body allows the beetles to refill the glands after depletion at least twice (Pasteels *et al.*, 1992). This ability could buffer a heterogeneity of secretion composition which would be expected from plant heterogeneity in PA content, if the beetles move from plant to plant in nature. The latter is supported by the observation that the secretion of beetles collected in nature sometimes contain PAs not found in the plant on which they were collected and fed on in the laboratory. For example senecionine and platyphylline were found in beetles collected on *S. fuchsii* (Table 2) (Rowell-Rahier *et al.*, 1991; Pasteels *et al.*, 1995). This plant does not contain these PAs, but they are found in the leaves of *Adenostyles alliariae* occurring in the same strand.

Larvae

The larvae of *Oreina* lacks defensive glands. PAs are sequestered in the body at the level of 61 µg/fourth instar larva (Dobler and Rowell-Rahier, 1994a). Feeding experiments with [^{14}C]senecionine N-oxide demonstrated that most of the PAs were sequestered in the hemolymph (60%) and the integument (28%). At best trace amounts of labelled PA were observed in the exuviae (Ehmke *et al.*, in prep.).

The gut of larvae shows similar permeability to PAs as that of adults. Foreign PAs (absent in normal food plant) such as monocrotaline N-oxide and heliotrine N-oxide are accumulated to the same extent as seneciphylline N-oxide, heliotrine being demethylated to rinderine (Hartmann *et al.*, in prep.). However, contrary to the observation made in the adults (see above), sequestration is rather transient. Sequestered [^{14}C]senecionine N-oxide is progressively lost with time (about half in third instar larvae after 48 h without molt) (Ehmke *et al.*, in prep.). Larvae rely more on the continuous intake of PAs from their food-plant than do the adults. Of course, continuous feeding is more expected in larvae than in adults.

O. cacaliae is larviparous. No PAs were detected in neonate larvae, but were present after one day of feeding. It seems therefore that females do not transfer sequestered PAs to their offspring (Dobler and Rowell-Rahier, 1994a).

Sequestration in species secreting autogenous cardenolides

Although the adults of *O. speciosissima* are able to secrete autogenous cardenolides, sequestration of PAs in their defensive secretion is as efficient in this species as in *O. cacaliae*, as demonstrated by feeding the beetle with [^{14}C]senecionine N-oxide (Ehmke *et al.*, 1991). The secretion of cardenolides seems to be reduced in sequester-

ing beetles feeding on *A. alliariae* (3.0 µg/secretion) compared to those feeding on *Petasites paradoxus* devoid of PAs in its leaves (5.7 µg/secretion). Since the amount of secretion produced is larger in sequestering beetles, the differences in concentration of cardenolides in the secretion is even higher (0.034 mol/l in sequestering beetles versus 0.111 in non-sequestering beetles) (Rowell-Rahier *et al.*, 1991, and unpublished results). This suggests that sequestration of PAs could compete with the production of cardenolides in the same glands.

PAs were also observed in the secretions of *O. intricata* and of *O. elongata* (from the Vallée des Merveilles) in admixture with cardenolides (Table 1, Pasteels *et al.*, 1995). Interestingly, no cardenolides were found in the adult secretion of *O. elongata* from the Col du Lautaret which exclusively fed on PA plants (*Adenostyles alliariae* and *A. alpina*), although the larvae from the same site produced cardenolides (Dobler and Rowell-Rahier, 1994a). In the Vallée des Merveilles, *O. elongata* was found in mixed stands of *Adenostyles leucophylla* (a PA plant) and *Circium spinosissimum* (a non-PA plant). The beetles were observed feeding on both plants often intimately intermingled. It remains to be tested, however, whether the absence of cardenolides in beetles from the Col du Lautaret is due to the competition of PA sequestration with cardenolide production in the glands of the adult beetles which continuously ingest PAs with their food.

As in *O. cacaliae*, larger amounts of PAs are sequestered in the body than in the secretion. Whereas only a few µg/secretion of PAs were detected in the three species, about 25 µg/beetle were detected in the bodies of *O. intricata* and *O. speciosissima* collected in Tschiertschen on *Senecio nemorensis* and up to 247 µg/beetle in *O. elongata* collected on *Adenostyles leucophylla* in the Vallée des Merveilles. In *O. elongata* the refilling of the glands after depletion with PAs stored in the body is much faster than the neosynthesis of cardenolides in beetles fed on *Circium spinosissimum* devoid of PAs (16.3 µg/secretion of PAs after nine days, but only 0.04 µg ouabaine equivalent/secretion of cardenolides, Pasteels *et al.*, 1995). This suggests again that sequestration of PAs in adult glands can supplant the autogenous production of cardenolides.

Preliminary experiments suggest that sequestration in *O. speciosissima*, *O. elongata*, and *O. intricata*, is as specific as in *O. cacaliae*, and that the gut appears to be more permeable than the gland cells' membrane. For example as in *O. cacaliae*, monocrotaline N-oxide is stored in the body of *O. speciosissima*, but not in its secretion (Rowell-Rahier *et al.*, 1991). Some differences were observed between the different species collected in the field or even between different populations of the same species, but these are difficult to interpret without detailed experiments under controlled conditions, considering the chemical heterogeneity of the plants, the ability of the beetles to move from plant to plant in nature, and the sometimes different range of host-plants of the different *Oreina* species. However, *Oreina elongata* from 3 locations (Col du Lautaret, Petit St Bernard and Vallée des Merveilles) contained in their secretion (from 12 to 31% of PA content) and body (circa 45%) a new non-plant PA, designated as oreine (Pasteels *et al.*, 1995). This compound was never observed in the other sequestering beetles even when fed with the same plants (*Adenostyles* spp.) Oreine is an epoxide of seneciphylline which most likely is formed by the beetle during uptake and sequestration of host-plant seneciphylline (Hartmann *et al.*, in prep.). Oreine was not observed in *O. elongata* collected at the Mattmark Dam and fed with *Adenostyles alliariae*. According to Kühnelt (1984), the beetles from Mattmark Dam belong to another subspecies than the beetles from the three other localities (respectively *O. elongata ruffoi* Franz and *O. elongata occidentalis* Ruffo). Examples of the sequestration ability of these three species are given in Table 3. In *O. speciossisima*, no PA is present

Table 3. PA composition (%) in the secretions of *O. speciosissima*, *O. intricata* and *O. elongata*

Alkaloids*	*O. speciosissima*		*O. intricata*		*O. elongata*	
	S	B	S	B	S	B
Senecionine	–	–	–	tr	56.0	11.2
Seneciphylline	59.0	tr	83.8	56.3	14.5	41.1
Doronenine	41.0	99.0	16.2	43.7	–	–
Spartioidine + platyphylline	tr	tr	tr	tr	tr	1.9
Bulgarsenine	tr	tr	–	tr	–	–
Oreine	–	–	–	–	29.4	45.8

S: secretion, B: body
*: Exclusively present as N-oxides.
O. speciosissima and *O. intricata* were collected in Tschiertschen in a mixed stand of *Senecio nemorensis*, *Adenostyles alliariae* and *Petasites paradoxus*; *O. speciosissima* on *P. paradoxus*; *O intricata* on *S. nemorensis*.
PA composition of *A. alliariae* and *S. nemorensis* is given in Table 2.
O. elongata was collected in the Vallée des Merveilles on *Adenostyles leucophylla* (PA composition in %: senecionine, traces; seneciphylline, 96.5; spartioidine + platyphylline, 3.5).
Modified from Pasteels *et al.* (1995).

in the leaves of the plant on which the beetle was collected, *P. paradoxus*, and the sequestered PAs must originate from other plants in the field (*A. alliariae* and *S. nemorensis*). As in *O. cacaliae*, only N-oxides are stored by the three species.

PAs were also reported in the fourth instar larvae of *O. speciosissima* (18 µg/larva), *O. intricata* (28 µg/larva) and *O. elongata* (10 µg/larva) fed with *A. alliariae* (Dobler and Rowell-Rahier, 1994a). The low content of PAs in the larvae of *O. elongata* compared to that found in the adults (247 µg/adult) is surprising even though the samples came from different populations (Col du Lautaret for the larvae and Vallée des Merveilles for the adults) and the analytical methods were different (respectively spectrophotometry of 3,5-dinitrobenzoic acid derivatives and gas chromatography after reduction). Further analyses are needed before reaching a firm conclusion on the sequestration ability of larvae and adults of this species.

Cardenolides are also present in the body of fourth-instar larvae of the three species (5.8 µg/larva in *O. speciosissima*, 12.6 in *O. intricata* and 3.2 in *O. elongata*). The exact location of the cardenolides in the larvae remains to be discovered, but in *O. gloriosa* (a non-sequestering species) cardenolides were found both in the hemolymph (one third of the amount) and tissue (unspecified) (Dobler and Rowell-Rahier, 1994a).

Finally in the oviparous species *O. elongata*, females transfer sequestered PAs and autogenous cardenolides to their eggs (respectively 4.5 µg/egg and 0.2/0.5 µg/egg), contrasting with the absence of female transfer of sequestered PAs to their offspring in the larviparous *O. cacaliae* (Dobler and Rowell-Rahier, 1994a). Neonate larvae of the larviparous species *O. speciosissima* and *O. intricata* were not analyzed.

Evolutionary and ecological aspects

Which of the three kinds of defensive chemistry is the most 'efficient' and which is ancestral in the genus? The first question cannot simply be answered by assessing the level of protection offered by the three different chemical defenses. Efficiency can only be measured in terms of increased fitness and trade-offs occur between the vari-

ous components of fitness. The second question can tentatively be answered by establishing independent phylogenies on which the modes of defense can be mapped. This allows speculation on sounder ground on the evolution of defensive patterns in an ecological context.

Two independent phylogenies of the genus *Oreina* have been established. The first was based on allozyme electrophoresis and used two *Chrysolina* species as outgroups (Fig. 1) (Dobler *et al.*, in revision). The second was based on mtDNA sequences (Hsiao *et al.*, in prep.). The latter included several *Chrysolina* species in the analysis and used another Chrysolinina genus (*Ambrostoma*) as outgroup. No phylogeny based on a restricted set of characters can be considered as definitive and the different methods lead to somewhat conflicting hypotheses, but some conclusions or hypotheses are relevant in this context.

The mtDNA phylogeny indicates that the genus *Chrysolina* is paraphyletic. The genus *Oreina* belongs to the same clade. *Oreina* and *Chrysolina* were considered as belonging to a single genus by some authors (for example, Bechyné, 1952; Bourdonné and Doguet, 1991). Alternatively, the genus *Chrysolina* should be splitted into differ-

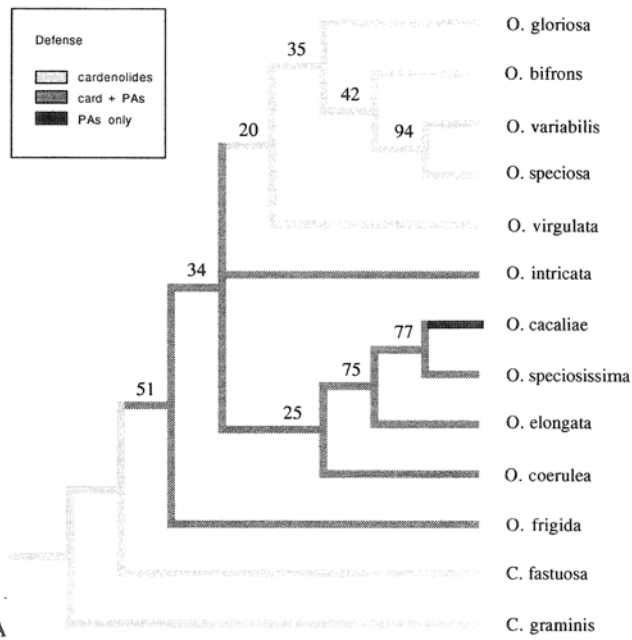

Fig. 1A. Legend see next page.

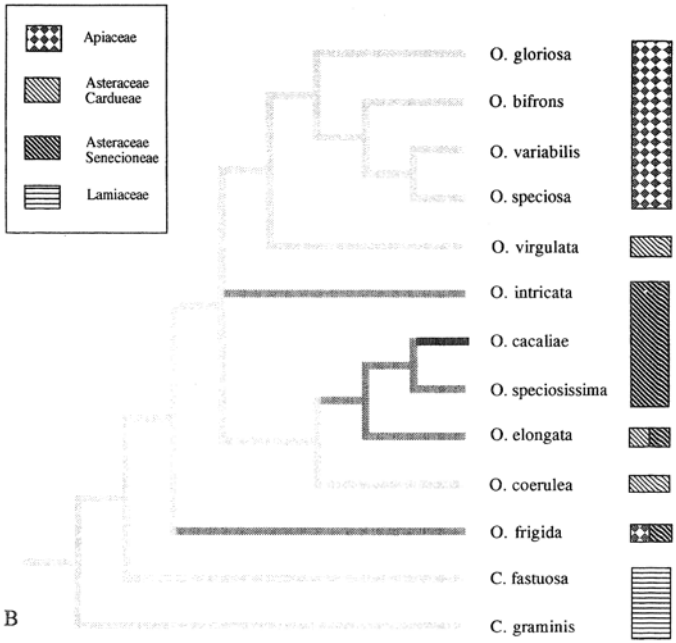

Fig. 1. Two hypotheses on the evolution of chemical defense in *Oreina*. The cladogram is a strict consensus of two maximum parsimony trees based on allozyme data (loci coded as characters). Two *Chrysolina*, *C. fastuosa* and *C. graminis* were used as outgroups. Beetles species and bootstrap values (500 samples) are given in A, an host-plant affiliation in B.
Modified from Dobler *et al.* (in revision).

ent genera. The secretion of autogenous cardenolides is ancestral in the genus *Chrysolina sensu lato*, and sequestration of PAs a derived condition (apomorphic) which occurred in *Oreina* species having colonized Senecioneae.

Feeding on Asteraceae would be plesiomorphic in the taxon *Oreina*. This is supported by both allozyme and mtDNA data. The precise evolutionary sequences leading to the present status of defensive chemistry in *Oreina* species are more uncertain.

One evolutionary scenario (allozyme data, Fig. 1A) suggests that sequestration of PAs in conjunction with the synthesis of autogenous cardenolides might be plesiomorphic. Accordingly, sequestration of PAs would have been secondarily lost in beetles which shifted to the Apiaceae or the Cardueae. In one species, *O. cacaliae*, it is the production of cardenolides which was lost and only sequestration selected. Another scenario is equally supported (Fig. 1B). Autogenous synthesis of cardenolides would be ancestral and the mixed chemical defense found in beetles having shifted on Sene-

cioneae. This would have occurred several times independently. The phylogeny based on mtDNA sequences gives a somewhat different branching pattern (not shown). It favours the second scenario, but a reverse evolution to cardenolide secretion from mixed defense would have occurred in *O. virgulata* (Germar). In all phylogenies (allozymes and mtDNA), the loss of cardenolides in *O. cacaliae* is secondary and evolved from a mixed chemical defense.

Hypotheses on the phylogeny of the *Oreina-Chrysolina* complex need further studies by the analysis of additional characters, but evidence suggests that different evolutionary events took place: the evolution of the mixed chemical defense from autogenous defense can be followed by pure sequestration, but also a return to pure autogenous defense seems possible in some species. The only evolution which appears unlikely is the reverse evolution to the ancestral secretion of autogenous cardenolides from pure sequestration of plant PAs.

Clearly the evolution of chemical defense is not towards a single 'best' solution. How do we understand the selective advantages of the different defenses and the ecological constraints acting on their evolution? Several aspects must be considered: defensive efficiency against natural enemies, reliability and flexibility of defense, cost of defense and host-plant availability.

Both cardenolides and PAs are well-known plant toxins deterring non-adapted herbivores (see Malcolm, 1991, for a review of the ecological functions of cardenolides in plants, and Hartmann and Witte, 1995, for the roles of PAs in plants). Besides, both classes of compounds protect insect herbivores against generalist natural enemies (for example, cardenolides in the monarch butterfly and other insects, Brower and Glazier, 1975; review in Malcolm, 1991; and PAs in various butterflies, Brown, 1984; review in Hartmann and Witte, 1995). Adults of *Oreina cacaliae* are avoided by naive shrews and larvae deter ants (Rowell-Rahier and Pasteels, 1990). This protection is due in part to sequestered PAs. The latter is supported by the observation that larvae of *O. speciosissima* fed without PAs (on *P. albus*) were better accepted by ants, than those of sequestering *O. cacaliae* fed on *A. alliariae*. Both kinds of larvae are very similar in many other ways.

Only one study was devised to compare the defensive efficiencies of autogenous cardenolides (in *O. gloriosa*) and sequestered PAs (in *O. cacaliae*) (Rowell-Rahier *et al.*, 1995). Similar morphs of the beetles were selected and offered in random order to birds after depletion or not of their defensive glands. A North-American bird was used as predator, the red-winged blackbird, *Agelaius phoeniceus*. Wild-caught birds were naive, having never encountered a European *Oreina*. Results indicate that cardenolide defense offers protection. Beetles with emptied glands were better accepted than beetles with full glands. *O. cacaliae* sequestering PAs in the secretion and body were even less palatable to the birds. As in the other species, beetles with emptied glands induced less rejection than those with full glands, but these beetles were still more rejected than *O. gloriosa* with their glands loaded with cardenolides. PAs provide the beetles with better protection from the birds than do cardenolides. This is an attractive conclusion explaining why pure sequestration was selected in *O. cacaliae* over autogenous cardenolide production. A strong defense also could explain the wide distribution and abundance of *O. cacaliae* compared to those of other *Oreina* species. Caution is needed, however, before reaching such a conclusion, based on experiments made with a single predator allopatric to the beetles.

Unfortunately, very little is known about the natural enemies of *Oreina* species, and nothing about their local distribution and abundance or about their susceptibility to PAs and cardenolides. Efficiency in defense is even more difficult to assess, as differ-

ent species of *Oreina* with different defenses often cohabit. Müllerian-Batesian mimicry could operate. Besides, the abundance of a species is not a strong indication of its level of immunity to natural enemies and other mechanisms than the selection pressure of natural enemies can explain the evolution of pure sequestration in *O. cacaliae* (see below).

At first sight, host-derived defense seems less costly (metabolic cost) and *de novo* synthesis of cardenolides, as it uses pre-synthesized plant compounds. However, there is no evidence for differential costs between the various modes of defense, and sequestration by itself could incur metabolic cost (review in Rowell-Rahier and Pasteels, 1992, see below). It was also suggested that autogenous defense is more reliable than sequestration of plant compounds, considering the variability in host-plant secondary chemistry (Brown and Francini, 1990). As stated above, long-term storage of PAs in the body of adult *Oreina* could temper plant chemical heterogeneity. Moreover, autogenous defense is not quantitatively and qualitatively uniform, even in individuals of the same population. Cardenolide patterns in *O. gloriosa* have a strong genetic component. They vary between sex and according to the beetle physiology and phenology (age, reproductive status, season) (Eggenberger *et al.*, 1992; Eggenberger and Rowell-Rahier, 1992, 1993).

A possible advantage of the mixed chemical defense is its flexibility, allowing the beetles to increase their range of host-plants. Populations of both *O. speciosissima* and *O. elongata* were found in which one or other defenses were expressed (Table 1), depending on available host-plants. *O. elongata* from Mattmark Dam produced only cardenolides, feeding in patches where the non-PA plant *Circium spinosissimum* is solely available. *O. speciosissima* from La Lécherette produced only cardenolides, *Petasites albus* (devoid of PAs in its leaves) being by far the dominant food plant in that locality. Conversely, *O. elongata* from the Col du Lautaret and *O. speciosissima* from Zinal fed exclusively on PA plants, *Adenostyles* spp., and only sequestered PAs (see above). However, plant availability is not the only basis of food-plant selection in nature. In the Black Forest (Germany) a population of *O. speciosissima* was found in a stand where both *P. albus* and *A. alliariae* were equally abundant and intermingled. *O. speciosissima* was found mainly on *P. albus*, and *O. cacaliae* on *A. alliariae*. The secretion of *O. speciosissima* contained large amounts of cardenolides, and at best traces of PAs. Although the possibility of sequestration was present, the beetles did not use it. Laboratory experiments demonstrated that larval relative growth rate in *O. speciosissima* was significantly higher on *P. albus*, than on *A. alliariae*. Feeding on *A. alliariae* induces some metabolic cost in *O. speciosissima* (Rowell-Rahier *et al.*, 1991). In the same way, larval growth was found to be better in *O. elongata* from the Col du Lautaret and from Mattmark Dam, when fed on *Circium spinosissimum* (without alkaloids) than on *A. alliariae* (a PA plant). This was especially so in the Mattmark Dam population that never naturally encountered PAs (Dobler and Rowell-Rahier, 1994b). Whether this trade-off between growth and sequestration results from the cost of handling PAs remains to be discovered.

Finally, the secondary loss of cardenolides in *O. cacaliae*, and the possible secondary loss of PA sequestration in species feeding on Apiaceae, were not necessarily a consequence of the selective advantages of pure sequestration or pure autogenous defense. If sequestration physiologically supplants cardenolide production in the defensive glands to the point that autogenous defense is not expressed anymore in beetles feeding exclusively on PA plants (see above), no selective force can act to maintain this trait. This is also true for the maintenance of a sequestration capacity in beetles feeding exclusively on plants with no PAs. Thus loss of these defensive capacities could become

constitutive by genetic drifts, or if defense incurs a load when not expressed (Pasteels *et al.*, 1995).

In conclusion, the evolutionary outcome seems to depend primarily on the plants on which the beetles specialized. None of the three defensive patterns seems to offer overwhelming advantages compared to the other. The evolution of host-shifts in herbivorous insects is a much debated issue (review in Futuyma and Keese, 1992) beyond the scope of this paper. Available evidence suggests that the selected chemical defense is a consequence of host-shifts during evolution, but does not suggest that the beetles moved to new host-plants to obtain additional host-derived protection. This is not a definitive conclusion, because it is based on indirect evidence in the absence of any assessment of the selective pressure exerted by foes in natural conditions. Paradoxically, the selective pressure of natural enemies remains the least understood factor acting on the evolution of chemical defense in *Oreina* species.

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