Published in Journal of Chemical Ecology 21, issue 8, 1163-1179, 1995

DISTRIBUTION OF AUTOGENOUS AND HOST-DERIVED CHEMICAL DEFENSES IN *Oreina* LEAF BEETLES (COLEOPTERA: CHRYSOMELIDAE)

JACQUES M. PASTEELS,[1,*] SUSANNE DOBLER,[2] MARTINE ROWELL-RAHIER,[2] ADELHEID EHMKE,[3] and THOMAS HARTMANN[3]

[1]*Laboratoire de Biologie animale et cellulaire, C.P. 160/12*
Université libre de Bruxelles
50 av. F.D. Roosevelt, B-1050 Brussels, Belgium

[2]*Zoologisches Institut der Universität Basel*
Rheinsprung 9, 4051 Basel, Switzerland

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

Abstract—The pronotal and elytral defensive secretions of 10 *Oreina* species were analyzed. Species feeding on Apiaceae, i.e., *O. frigida* and *O. viridis*, or on Cardueae (Asteraceae), i.e., *O. bidentata*, *O. coerulea*, and *O. virgulata*, produce species-specific complex mixtures of autogenous cardenolides. *O. melanocephala*, which feeds on *Doronicum clusii* (Senecioneae, Asteraceae), devoid of pyrrolizidine alkaloids (PAs) in its leaves, secretes, at best, traces of cardenolides. Sequestration of host-plant PAs was observed in all the other species when feeding on Senecioneae containing these alkaloids in their leaves. *O. cacaliae* is the only species that secretes host-derived PA N-oxides and no autogenous cardenolides. Differences were observed in the secretions of specimens collected in various localities, because of local differences in the vegetation. The other species, such as *O. elongata*, *O. intricata*, and *O. speciosissima*, have a mixed defensive strategy and are able both to synthesize de novo cardenolides and to sequester plant PA N-oxides. This allows a great flexibility in defense, especially in *O. elongata* and *O. speciosissima*, which feed on both PA and non-PA plants. Populations of these species were found exclusively producing cardenolides, or exclusively sequestering PA N-oxides, or still doing both, depending on the local availability of food-plants. Differences were observed between species in their ability to sequester different plant PA N-oxides and to transform them. Therefore sympatric species demonstrate differences in the composition of their host-derived

*To whom correspondence should be addressed.

secretions, also resulting from differences in host-plant preference. Finally, within-population individual differences were observed because of local plant heterogeneity in PAs. To some extent these intrapopulation variations in chemical defense are tempered by mixing diet and by the long-term storage of PA N-oxides in the insect body that are used to refill the defensive glands.

Key Words—*Oreina* spp., Coleoptera, Chrysomelidae, Asteraceae, Senecioneae, Cardueae, Apiaceae, chemical defense, cardenolides, pyrrolizidine alkaloids, sequestration.

INTRODUCTION

Adult *Oreina* leaf beetles are chemically protected by a secretion oozing from elytral and pronotal glands. Such glands are characteristic of leaf beetles belonging to the Chrysomelinae and some other leaf beetle taxa (reviewed in Pasteels et al., 1988a, 1994). Although only six *Oreina* species have been previously studied, these can be classified into three categories according to their defensive chemistry, exemplifying different defensive strategies.

First *O. gloriosa*, feeding exclusively on *Peucedanum ostruthium* (Apiaceae), relies for its defense on de novo synthesis of cardenolides from a sterol precursor (Van Oycke et al., 1988; Eggenberger et al., 1992). The mixture of cardenolides produced is complex and shows high interindividual variability. Individual variation is in part correlated with physiological factors such as age and mating status (Eggenberger and Rowell-Rahier, 1993), but a strong heritability of the proportions of compounds was demonstrated by full-sib and mother–offspring correlations (Eggenberger and Rowell-Rahier, 1992). Significant differences in the proportions of the cardenolides making up the defensive mixtures also were observed between different alpine populations. These differences were correlated with the genetic distances (estimated from allozyme data) and the geographic distances separating the populations (Eggenberger and Rowell-Rahier, 1991). Three other species, *O. bifrons*, *O. speciosa*, and *O. variabilis*, feeding on Apiaceae, similarly secrete mixtures of cardenolides. The average mixtures are species specific, and their level of similarity correlates with the genetic distances between the species (allozyme electrophoresis) (Rowell-Rahier and Pasteels, 1994). Thus, chemical defense is able to evolve rapidly, possibly in part by genetic drift. In this context, it should be noted that *Oreina* species apparently form metapopulations, the genetic structure of which has been analyzed in two species, *O. cacaliae* and *O. speciosissima* (Rowell-Rahier, 1992). The secretion of cardenolides is considered to be plesiomorphic in the genus, since it is frequently observed in other genera belonging to the same subtribe Chrysolinina (i.e., in the genera *Chrysolina* and *Ambrostoma*) or to a related subtribe, the Doryphorina (genera *Calligrapha* and *Zygogramma*) (Pasteels et al., 1994).

Secondly, *O. cacaliae*, feeding exclusively on members of the Senecioneae (Asteraceae), secretes sequestered plant pyrrolizidine alkaloid N-oxides (PA N-oxides) for defense (Pasteels et al., 1988b; Ehmke et al., 1991). In the beetles studied from the Vosges (France) and the Black Forest (Germany), only PA N-oxides from *Adenostyles alliariae* were sequestered in the defensive secretion, but not PAs from *Senecio fuchsii*, another food plant in the field. PAs from both plants, however, were sequestered in the body (Rowell-Rahier et al., 1991).

Finally, *O. speciosissima*, also feeding on Senecioneae, has a mixed defensive strategy, being able to synthesize cardenolides and to sequester PAs. Interestingly, although the beetles readily sequestered PAs when fed with *A. alliariae* in the laboratory, pooled secretions of beetles collected from two different localities contained, at best, trace amounts of PAs (Rowell-Rahier et al., 1991). In the field, these beetles fed mainly on *Petasites albus*, which is devoid of PAs in its leaves, although *A. alliariae* was equally frequent in one locality. The mixed strategy possibly illustrates an intermediate evolutionary stage, but the flexibility it allows could be selectively advantageous in its own right.

Our ultimate goal is to understand the evolution of chemical defense in the genus. Independent phylogenies based on molecular data will be presented elsewhere. Here, we will focus on the distribution of the three defensive strategies within the genus (i.e., de novo synthesis, sequestration, and mixed strategy) and limit ourselves to adult defense. Larval defense is described by Dobler and Rowell-Rahier (1994a) and Ehmke et al. (in preparation). In the present paper, 10 species will be examined (*O. cacaliae*, *O. speciosissima*, and eight previously unstudied species), bringing the total of species studied to 14, i.e., the majority of species occurring in the Swiss and French Alps. Two main questions will be addressed: How are the different strategies linked to host-plant affiliation, i.e., Apiaceae or Asteraceae, and among the latter, Senecioneae or Cardueae? In sequestering species, how is the defensive chemistry influenced by the local host-plant community? The emphasis will be on the defensive chemistry of beetles collected in the field. Laboratory feeding experiments with a specific food plant will be described only when necessary to confirm the beetles' ability for sequestration.

METHODS AND MATERIALS

Collecting Sites and Host Plants

Unless specified otherwise host-plant affiliations are based on direct observations of feeding beetles. Beetle nomenclature follows Bourdonné and Doguet (1991), and plant nomenclature, Flora Europaea. The species with their host plants are listed in Table 1.

TABLE 1. DISTRIBUTION OF DE NOVO SYNTHESIZED CARDENOLIDES AND SEQUESTERED PAs IN DEFENSIVE SECRETIONS OF *Oreina* spp.

Species	Defensive compound	Host plants	Localities
O. viridis	cardenolides	*Meum athamanticum* (Apiaceae)	Tschiertschen
O. frigida	cardenolides	*Meum athamanticum* (Apiaceae)	Hörnlihütte, Engstligenalb
O. bidentata	cardenolides	*Leuzea rhapontica* (Asteraceae-Cardueae)	Bosco Gurin
O. coerulea	cardenolides	*Centaurea nemoralis* (Asteraceae-Cardueae)	Calmbach, Bad Liebenzell
O. virgulata	cardenolides	*Cirsium spinosissimum* (Asteraceae-Cardueae)	Engstilgenalb, Hörnlihütte
O. melanocephala	cardenolides ?[a]	*Doronicum clusii* (Asteraceae-Senecioneae)	Hörnlihütte
O. cacaliae	PAs	*Adenostyles alliariae*, *Senecio nemorensis* (Asteraceae-Senecioneae)	Tschiertschen
O. intricata	cardenolides PAs	*Adenostyles alliariae*, *Senecio nemorensis*, (Asteraceae-Senecionae)	Tschiertschen
O. speciosissima	cardenolides PAs	*Petasites albus*, *Senecio nemorensis* (Asteraceae-Senecioneae)	Tschiertschen
	PAs	*Adenostyles alliariae* (Asteraceae-Senecioneae)	Zinal
O. elongata	cardenolides PAs	*Adenostyles leucophylla*, *Cirsium spinosissimum* (Asteraceae-Senecionae, Cardueae)	Vallée des Merveilles
	PAs	*Adenostyles alliariae*, *A. alpina* (Asteraceae-Senecioneae)	Col du Lautaret
	cardenolides	*Cirsium spinosissimum* (Asteraceae-Carduae)	Mattmark dam

[a] At best traces; needs confirmation.

Species Feeding on Apiaceae. O. frigida (Weise) was collected at Hörnlihütte above Arosa (Graubünden, Switzerland, 2600 m) and Engstligenalb (Berner Oberland, Switzerland, 2000 m). This species was found under stones at the first location and on *Cirsium spinosissimum* (L.) Scop. (Asteraceae, Cardueae) at the second. In the laboratory, it fed on *Meum athamanticum* Jacq.

(Apiaceae), but also on *Adenostyles alliariae* (Gouan) Kern. (Asteraceae, Senecioneae) and not on *C. spinosissimum*, *Doronicum clusli* (All.) Tausch, or *Petasites albus* (L.) Gaertn. (both Asteraceae, Senecioneae). The food plant of this species was not yet reported.

O. viridis (Duftschmid) was collected at Tschiertschen (Graubünden, Switzerland, above the tree line, 2000 m) under stones. The host plant reported in the literature is *M. athamanticum* (Bourdonné and Doguet, 1991), which is accepted in the laboratory as are other Apiaceae, but no Asteraceae.

Species Feeding on Asteraceae. O. bidentata Bontems was collected at Bosco Gurin (Tessin, Switzerland, 1600 m) on *Leuzea* (=*Centaurea*) *rhapontica* (L.) J. Holub (Asteraceae, Cardueae).

O. cacaliae (Schrank) was collected at Tschiertschen on *A. alliariae* and *Senecio nemorensis* L. (s.str.) (Asteraceae, Senecioneae).

O. coerulea (Olivier) was collected at Calmbach (Black Forest, Germany, 550 m) and Bad Liebenzell (Black Forest, Germany, 550 m) both on *Centaurea nemoralis* Jordan (Asteraceae, Cardueae).

O. elongata (Suffrian) was collected at Col du Lautaret and Col du Petit St. Bernard. (near Briançon, France, 2000 m) on *A. alliariae* and *Adenostyles alpina* (=*glabra*) (L.) Bluff & Fingerh.; Mattmark dam (Valais, Switzerland, 2400 m) on *C. spinosissimum*; and Vallée des Merveilles (Parc National du Mercantour, France, 2500 m) on *Adenostyles leucophylla* (Wild.) Reichenb. and *C. spinossissimum*.

O. intricata (Germar) was collected at Tschiertschen on *S. nemorensis*.

O. melanocephala (Duftschmid) was collected at Hörnlihütte. This species was found under stones, close to *D. clusii*. The beetles fed on *D. clussi*, but not on other Asteraceae or Apiaceae present in the same location. *Doronicum* spp. are the host plants reported in the literature for this *Oreina* by Bourdonné and Doguet (1991).

O. speciosissima (Scopoli) was collected at Tschiertschen mostly on *P. albus*; and occasionally on *S. nemorensis* and *A. alliariae*, and at Zinal (Valais, Switzerland, 1800 m) on *A. alliariae*.

O. virgulata (Germar) was collected at Engstligenalb and Hörnlihütte on *C. spinosissimum*.

Detection and Quantification of Cardenolides in Secretion

For most species, secretions of five or more beetles were collected on pieces of filter paper or in glass capillaries and pooled in methanol. Only two specimens of *O. frigida* and three of *O. melanocephala* were available for study. The methanol extract of pooled secretions was evaporated under vacuum, the residue redissolved in acetonitrile–water 1 : 10, and analyzed after filtration by reverse-phase HPLC [two pump system (Waters 510); detector: photodiode array (Waters

994), 220 nm; column: Macherey-Nagel cartridge, C18, 3 mm, 4 × 130 mm; eluent: chromatography grade acetonitrile (Baker) and water (Merck), 15–42% acetonitrile linear in 36 min, 0.45 ml/min; data analysis system: Maxima 820 data station]. Cardenolides were recognized by their UV spectra. For quantitative analyses, known volumes of secretion were processed and 2 mg ouabain was used as internal standard during HPLC analysis. Quantification of cardenolides was based on peak area measurements and expressed in milligram ouabain equivalents.

Alkaloid Extraction

Individual beetles previously kept frozen at −20°C were ground with quartz sand in 4 ml acidic MeOH (1% HCl) in a mortar for 10 min. After centrifugation the supernatant was divided into two aliquots, which were evaporated under reduced pressure. One aliquot was redissolved in 2 ml of dilute NH_4OH and directly applied to an Extrelut column (3 ml, Merck). PAs were eluted with 20 ml of CH_2Cl_2. After evaporation, the residue was redissolved in 0.1–0.5 ml MeOH. This extract provided the fraction occurring as tertiary PAs in the beetle. To obtain the fraction of total PAs (tertiary PAs and PA N-oxides), the remaining aliquot was redissolved in 2 ml of 0.1 M H_2SO_4 and mixed with Zn dust in excess. The mixture was stirred at room temperature for 4 hr, then the solution was made alkaline and treated as described above. Methanolic extracts of secretions were evaporated to dryness and the residue redissolved in 5 ml of 1 M HCl. Aliquots of 2 ml each were treated to reduce PA N-oxides in tertiary PAs as described above.

Plant leaves were preserved by freezing (−18°C) as soon as possible after harvesting in the field. Following freeze-drying, 0.5–1.0 g of the powdered material was extracted in 25 ml of 0.05 M H_2SO_4 for 3–4 min (Ultra-turrax) and left to stand for 30 min. After centrifugation, one part of the supernatant was adjusted to pH 11 with NH_4OH and applied to an Extrelut column as above. For total PA analysis, part of the acidic supernatant was adjusted to 0.25 M H_2SO_4, mixed with Zn dust, and further processed as described above.

Alkaloid Analysis

PAs were separated and analyzed quantitatively by capillary GC on quartz columns (WCOT, 15 × 0.25 mm DB-1, J & W Scientific) using a Hewlett Packard 5890 series II. Conditions: injector, 250°C; temperature program, 150–300°C at 6°C/min; split ratio, 1:20; injection vol., 1–2 μl; carrier gas, He 0.75 bar; detection, flame ionization and nitrogen detectors. Atropine was used as internal standard. The GC response factors of the major PAs analyzed in these experiments were shown to be almost identical. Retention indices were calculated from cochromatographed hydrocarbon standards.

GC-MS. A Carlo Erba Mega 5160 gas chromatograph, equipped with a quartz column (30 m × 0.32 mm) as specified above, was directly coupled to a quadripole mass spectrometer Finnigan MAT 4515. GC conditions were as specified above (Witte et al., 1993).

RESULTS

Species Secreting Cardenolides and No PAs. All species feeding on Apiaceae as well as those feeding exclusively on Cardueae secrete complex mixtures of cardenolides (Table 1). At least 14 cardenolides were observed in the secretion of *O. bidentata*, 15 in that of *O. coerulea*, 5 in *O. frigida*, 15 in *O. viridis*, and 19 in *O. virgulata*. The secretions of *O. coerulea* from both sites in the Black Forest are very similar, not only in the different compounds observed, but also in their proportions. By contrast, the secretion of *O. bidentata* differs markedly from that *O. coerulea* in the proportions of the constituents. The two species are morphologically very similar (Kühnelt, 1984), but the difference in the composition of the secretions confirms the separation into different species, as in general our analyses show that cardenolide mixtures offer discriminating taxonomic characters (Rowell-Rahier and Pasteels, 1994).

Interestingly, no PAs were observed in the secretion of *O. frigida*, although the beetles accepted *A. alliariae* in the laboratory. Either the beetle is unable to sequester PAs or it mainly fed on *M. athamanticum* in the field. Only a few beetles were available for study, and more experiments are needed to answer this question.

Similarly, PAs were not observed in the secretion of *O. melanocephala* feeding on *D. clusii*. Although PAs were observed in some *Doronicum* (e.g., *D. macrophyllum* and *D. pardalianches*) (Hartmann and Witte, 1994), no PAs were found in *D. clusii* (by TLC analysis of 2 g of dried leaves material). Intriguingly, no or only traces of cardenolides were observed in the few available secretions. More secretion of this rare species is needed for determining what it actually secretes.

Besides cardenolides, all these species may secrete ethanolamine and tyrosine betaine, as do the other previously studied *Oreina* (Pasteels et al., 1994). No attempt was made to identify unambiguously these constituents of unknown function, or to identify the structure of the different cardenolides detected in the secretions.

Species Secreting Sequestered PA N-Oxides and No Cardenolides. O. cacaliae is the only species that secretes host-plant-derived PAs but no autogenous cardenolides. Only PA N-oxides were observed in the beetles' secretions and stored in their bodies, confirming our previous observations (see above). For simplicity, PAs will be designated by their names, omitting the fact that they are in the form of N-oxides.

In Tschiertschen, the beetles were found on both *A. alliariae* and *S. nemorensis*, which grew in close proximity. The glands of wild caught specimens were experimentally emptied, and then the animals were fed for five days on one or the other host plant. After this feeding period, their secretions were collected and both the secretions and the beetles were analyzed for PAs (Table 2). The chemical defense of the beetles depended on the plant on which they fed, but did not exactly mirror food-plant chemistry. The beetles that fed on *A. alliariae* secreted and had in their bodies seneciphylline and senecionine, as did the beetles from the Vosges studied previously (Rowell-Rahier et al., 1991). However, contrary to the latter, they contained traces of other PAs, e.g., doronenine and bulgarsenine, which must have been acquired from *S. nemorensis* in the field. By contrast, beetles fed in the laboratory with *S. nemorensis* secreted

TABLE 2. RELATIVE ABUNDANCES OF PAS AND CONTENTS IN *O. cacaliae* AND ITS HOST PLANTS FROM TSCHIERTSCHEN

	Abundance (%)					
	O. cacaliae[a]					
	Fed on *A. alliariae*		Fed on *S. nemorensis*		Host plants	
	Secretion ($N = 8$)	Body ($N = 3$)	Secretion ($N = 7$)	Body ($N = 3$)	*A. alliariae*	*S. nemorensis*
Nemorensine						trace
Retroisosenine[b]	trace			trace		trace
Senecionine	20.9	10.7	trace	trace	3.4	
Seneciphylline	79.1	89.3	31.6	22.9	93.0	trace
Nemorensine isomer[b]						0.6
Doronenine	trace	trace	68.4	61.5		trace
Spartioidine + platyphylline[c]	trace	trace		trace	3.6	
Bulgarsenine	trace		trace	15.6		92.4
Intergerrimine					trace	
Neoplatyphylline				trace	trace	6.9
Total PAs μg/beetle; mg/g dry wt leaves	4.2	102.0	4.5	74.3	66.0	8.0

[a]The glands were emptied and the beetles fed for 5 days before the secretions were collected. *N*: number of pooled secretions or specimens.
[b]Tentative identification.
[c]Not separated in the analysis.

doronenine as the major constituent and also contained in their body significant amounts of bulgarsenine, the major constituent of *S. nemorensis*. Only traces of bulgarsenine were found in the secretions, however, and significant amounts of seneciphylline were observed in both bodies and secretions, possibly sequestered from *A. alliariae* in the field. These data demonstrate that the beetles selectively sequester (and probably metabolize to some extent) certain PAs more than others (Hartmann et al., in preparation). Heterogeneity in the plant community causes heterogeneity in beetle defense. It should be emphasized, however, that the analyses were performed on pooled secretions and the beetles fed for some time in the laboratory. Individual secretions and beetles from the field must be analyzed in order to perceive fully the variation in beetle defense due to plant diversity and heterogeneity.

Species Secreting Both De Novo Synthesized Cardenolides and Host-Derived PA N-Oxides. The mixed defensive strategy was observed in *O. speciosissima* as already reported (Rowell-Rahier et al., 1991), and also in *O. intricata* and *O. elongata* (Table 1). At least 13 cardenolides were observed in the secretion of *O. intricata*, but, as for all species secreting PAs, the presence of PA N-oxides interferes with the detection of cardenolides in HPLC. In Tschiertschen, the beetles fed mainly on *S. nemorensis*, and doronenine was found both in their secretion and their bodies (Table 3), as observed in *O. cacaliae* fed on *S. nemorensis*. However, when this beetle was observed on *A. alliariae* in the field, seneciphylline was sequestered in secretion and body. Both seneciphylline and doronenine were found in the body of one beetle. This beetle presumably had a mixed diet in the field. The body of another beetle contained 255.3 μg/g of seneciphylline and only traces of doronenine, showing that the PA composition may greatly depend on the diet in the field.

The mixed defensive strategy is best illustrated in *O. speciosissima* and *O. elongata*. *O. speciosissima* was observed in Tschiertschen mostly on *P. albus*, which is devoid of PAs in its leaves, but also to a lesser extent on *S. nemorensis* and *A. alliariae*. The beetles sequestered PAs from both *Adenostyles* and *Senecio*, suggesting that they had a mixed diet in the field, like *O. intricata* and probably *O. cacaliae*. In Zinal, *O. speciosissima* fed exclusively on *A. alliariae*. Preliminary analysis by HPLC of secretion from this location indicates that it contains, at best, traces of cardenolides, but the presence of sequestered PAs (characteristic of *A. alliariae*) was obvious.

Striking differences in chemical defense between populations as the result of local differences in the distribution of food plants were also observed in *O. elongata*. Beetles from Mattmark dam fed exclusively on *C. spinosissimum*; no other potential food plants were observed in the vicinity. As expected, they secreted only cardenolides (more than 15 compounds), as no PAs are present in *C. spinosissimum*. This patch of beetles probably has been isolated on *Cirsium* since the building of the dam in 1970. The beetles, however, have kept their

TABLE 3. RELATIVE ABUNDANCE OF PAs AND CONTENTS IN SECRETIONS AND BODIES OF *O. intricata* AND *O. speciosissima* FROM TSCHIERTSCHEN

	Abundance (%)[a]			
	O. intricata		*O. speciosissima*	
	Secretion (N = 8)	Body (N = 1)	Secretion (N = 12)	Body (N = 5)
Retroisosenine isomer[b]			trace	
Retroisosenine[b]		trace	trace	trace
Senecionine		trace	trace	
Seneciphylline	83.8	56.3	59.0	trace
Nemorensine isomer[b]		trace		
Doronenine	16.2	43.7	41.0	99.0
Spartioidine + platyphylline[c]	trace	trace	trace	trace
Bulgarsenine		trace	trace	
Neoplatyphylline		trace	trace	trace
Total content (μg/beetle)	7.4	25.0	8.3	25.3

[a] N: number of pooled secretions or specimens.
[b] Tentative identification.
[c] Not separated in the analysis.

ability to sequester PAs, as proved by feeding them on *A. alliariae* in the laboratory. In contrast, no cardenolides could be detected in the secretion of specimens from the Col du Lautaret, which fed on *A. alliariae*. The secretion contained, however, significant amounts of PAs (Table 4). When the beetles were fed for two weeks on *C. spinosissimum*, after their glands had been emptied before the experiment and again after one week, the renewed secretion at the end of the two weeks contained only PAs obviously mobilized from their body reservoir (Pasteels et al., 1992). Nevertheless, *O. elongata* from the Col du Lautaret do have the capacity to synthesize cardenolides, since cardenolides were found in their eggs and larvae (Dobler and Rowell-Rahier, 1994a). Finally, *O. elongata* collected in the Vallée des Merveilles on either *A. alliariae* or *C. spinosissimum* secreted both cardenolides and PAs. The beetles collected on *Cirsium* contained nearly as much PAs in their secretion and body as those collected on *Adenostyles*, demonstrating that they had also fed on *Adenostyles* (Table 4). A mixed diet in these beetles is not surprising, both plants are growing intimately intermixed in the field.

Interestingly, although the food plants of *O. elongata* contained seneciphylline as a major constituent (Table 5), the secretion and body of the beetles

TABLE 4. PA COMPOSITION AND CONTENT IN *O. elongata* AND IN ITS HOST PLANTS

	PA composition (%)[a]					
			Vallée des Merveilles			
			On *A. leucophylla*		On *C. spinosissimum*	
	Col du Lautaret on *A. alliariae* and *A. alpina*, secretion (N = 19)	Petit St Bernard on *A. alliariae*, secretion (N = 8)	secretion (N = 5)	body (N = 5)	secretion (N = 5)	body (N = 5)
Senecionine	9.8	43.6	56.0	11.2	66.8	11.9
Seneciphylline	58.8	34.5	14.5	41.1	13.3	43.3
Spartioidine + platyphilline[b]		9.4	trace	1.9	trace	trace
Intergerrimine		trace	trace	trace	trace	trace
Oreine	31.4	12.5	29.4	45.8	19.9	44.8
Total (μg/individual)	6.4	N.D.[c]	7.03	247.3	4.5	130.1
Concentration (M)	1.9×10^{-1}		1.4×10^{-1}		1.0×10^{-1}	

[a] N: number of pooled secretions or specimens.
[b] Not separated in the analysis.
[c] Not determined because of leachage during transport.

TABLE 5. PA COMPOSITION AND CONTENT IN LEAVES OF HOST PLANTS OF *O. elongata*

	PA composition (%)		
	A. alliariae	*A. glabra*	*A. leucophylla*
Senecionine	trace	trace	trace
Seneciphylline	92.0	94.8	96.5
Spartioidine + platyphylline[a]	8.0	5.2	3.5
Neoplatyphylline	trace		
Total (mg/g dry wt)	30.9	23.4	18.9

[a] Not separated in the analysis.

also contained significant amounts of senecionine and a new PA derivative, oreine (Table 4) (Hartmann et al., in preparation). A similar pattern of PAs was found in the secretion of beetles from a third population, collected in the Col du Petit St Bernard. This contrasts with the PA composition of secretion of *O. cacaliae* fed on the same host plant, where no oreine and less senecionine compared to seneciphylline was found. The amounts of PAs sequestered in the body of *O. elongata* are much larger than the amounts sequestered by the other *Oreina* species (compare data in Table 4 with those in Tables 2 and 3).

O. elongata collected in the Vallée des Merveilles on *Adenostyles* had an average of 3.5 μg (ouabain equivalent) of cardenolides/secretion ($N = 15$, pooled secretions); those collected on *Cirsium*, 2.7 μg ($N = 5$). This is somewhat less, but within the same order of magnitude, as the amount of PAs sequestered in the secretion (Table 4). However, sequestration of PAs in the glands seems to occur much faster than synthesis of cardenolides, as proved by the following experiment. The glands of beetles ($N = 10$) collected on *Adenostyles* were emptied. The beetles were then fed during nine days in the laboratory on *Cirsium* and their secretion collected again. The mean amount of cardenolides was only 0.04 μg (ouabain equivalent)/secretion, but that of PAs was 16.3 μg/secretion, although *Cirsium* does not contain PAs in its leaves. Clearly, PAs stored in the body can be mobilized in the glands before significant amounts of cardenolides could be synthesized.

DISCUSSION

Relation Between Host-Plant Use and Defensive Chemistry. All species studied here, except *O. cacaliae* and possibly *O. melanocephala*, secrete cardenolides. As in the other leaf beetles studied to date, complex mixtures of cardenolides are produced, the mixture being species specific.

PAs are sequestered from species of the tribe Senecioneae, and species feeding exclusively on Apiaceae or Cardueae only secrete autogenous cardenolides in addition to ethanolamine and tyrosine betaine. All species feeding on Senecioneae containing PAs seem able to sequester some plant PAs, sometimes after transformation. Only PA N-oxides are observed in beetles' secretions and stored in their bodies, confirming our previous observations (Ehmke et al., 1991).

Although ethanolamine and tyrosine betaine were not systematically searched for, so far tyrosine betaine was found in all species producing cardenolides with or without PAs, but is clearly absent in *O. cacaliae*, which does not secrete cardenolides. Ethanolamine was detected in all secretions in which it was looked for, not only in the genus *Oreina* (i.e., *O. cacaliae*, *O. gloriosa*, *O. speciosissima*), but also in the Chrysolinina and Doryphorina (Pasteels et al., 1988a, 1994).

Additional experiments are needed to determine if *O. frigida* and *O. melanocephala* conform to these general rules. First, *O. frigida* collected in the field did not secrete PAs, although it is able to feed on *Adenostyles* in the laboratory. The absence of PAs in this species could simply result from the fact that the few beetles collected did not feed on *Adenostyles* in the field or could be due to an inability to sequester. Second *O. melanocephala*, which fed on *Doronicum* devoid of PAs, did not secrete cardenolides or secreted only in trace amounts. More material of this rare species is needed for a better understanding of its secretion and for deciding with confidence if it has lost the ability to synthesize cardenolides, as did *O. cacaliae*.

Sources of Variation in Defensive Chemistry. As expected, important within-species differences are observed in those beetles that sequester PAs, because of local differences in the vegetation. The secretion of *O. cacaliae* from Tschiertschen contained a spectrum of PAs including compounds characteristic of both *A. alliariae* and *S. nemorensis*, whereas the secretion of specimens collected in the Vosges or the Black Forest contained only PAs from *A. alliariae* (Rowell-Rahier et al., 1991). In these localities, *S. nemorensis* is substituted by *S. fuchsii*, which, instead of macrocyclic PAs, contains open-chain mono- and diesters. The beetles from Tschiertschen sequestered PAs from *S. nemorensis* in both their bodies and secretions, whereas the beetles from the Vosges only sequestered PAs from *S. fuchsii* in their bodies, but not in their secretion. PAs from *A. alliariae* were sequestered in both the bodies and secretion of beetles from both localities.

Individual variation in sequestered defense within a population is also to be expected, since the various host plants with different secondary chemistry are distributed in patches of various sizes, and since beetles have low vagility for long periods of time (S. Knoll, unpublished observation). Our results indicate, however, that mixed diets occur in the field. Sequestration and storage of

large quantities of PAs for long periods occur in the body from which refilling the glands is possible. This could temper individual variation in host-derived defense (Pasteels et al., 1992).

Interpopulation variation in chemical defense is even more obvious in the beetles with the mixed defensive strategy, illustrating the flexibility of this mode of defense. In both *O. speciosissima* and *O. elongata*, populations were observed that practically only sequestered PAs or that secreted only autogenous cardenolides or that did both. Of course, this reflects local variation in host-plant distribution, but intrinsic genetic differences between beetles from distant populations adapted to local vegetation cannot be excluded, and has been suggested in *O. elongata* populations (Dobler and Rowell-Rahier, 1994b). As already stated, *Oreina* species form metapopulations and morphological differences between populations have been reported that justify for some taxonomists the subdivision of species into subspecies or even different species. For example, *O. speciosissima* from Zinal and other alpine populations differ from beetles from lower altitudes in details in their morphology to the point that they were considered as a distinct species: *O. perenei* (e.g., Freude et al., 1966). However, this distinction does not appear to be valid (for detailed taxonomic discussion, see Kühnelt, 1984). Morphological differences also exist between *O. elongata* from the Col du Lautaret, the Vallée des Merveilles, and Mattmark dam, possibly reaching subspecies level. The two first populations belong to *O. elongata occidentalis* Ruffo and the latter to *O. elongata ruffoi* Franz (e.g., Kühnelt, 1984).

The same exocrine glands that store and possibly synthesize autogenous cardenolides also store and possibly transform sequestered PAs. We do not know how these two processes interfere with each other, but it is tempting to speculate that sequestration competes with the secretion of cardenolides. At least in *O. elongata*, our data indicate that the refilling of the glands with sequestered PAs is much faster than with autogenous cardenolides. If sequestration competes with de novo synthesis, it could explain why beetles feeding exclusively for many generations on *Adenostyles* have their production of cardenolides suppressed. If this suppression becomes constitutive, a situation similar to that observed in *O. cacaliae* would be reached. This could occur by genetic drift the more easily as no selective force can act on the synthesis of cardenolides, which is not expressed anymore. Furthermore, experiments demonstrated that naïve red-winged blackbirds more often rejected *O. cacaliae*, which only sequesters PAs, than *O. gloriosa*, a species which is very similar in both size and color, but only produces cardenolides (Rowell-Rahier et al., 1995).

It would be too simplistic, however, to consider the production of both cardenolides and PAs as a transitory evolutionary step towards exclusive plant-derived defense. The advantages of one or the other defense is difficult to assess and depends not only on the distribution of natural enemies and their answer to

both kinds of compounds, but also on the distribution of plants and of their nutritional value. The mixed strategy is observed in three species, but only a single species relies solely on sequestered PAs.

Interspecific differences in host-derived defenses is, of course, even greater that intraspecific variation in both allopatric and sympatric situations. Not only do the different *Oreina* exhibit differences in their host-plant preferences, but they handle PAs from the same plant species in different ways. For example, *O. elongata* feeding on *A. alliariae* sequester higher proportions of senecionine compared to seneciphylline, than *O. cacaliae* or *O. speciosissima* feeding on the same plant, and above all produce significant quantities of oreine, which is not detected or is detected only as traces in the other species. Oreine is not present in *A. alliariae*, and it must be derived from seneciphylline (Hartmann et al., in preparation). Similarly, among PAs belonging to the same structural group (see Hartmann and Witte, 1994, for a classification of PAs) doronenine is the major PA found in *O. cacaliae*, *O. speciosissima*, and *O. intricata*, feeding on *S. nemorensis*. In *S. nemorensis*, the only possible source of such PAs, doronenine is not very abundant and bulgarsenine is far more abundant. Either these beetles are very selective in their sequestration, or they transform bulgarsenine into doronenine (Hartmann et al., in preparation).

Strong inter- and intraspecific variations in host-derived defense resulting from local heterogeneity in phytochemistry, differences in plant communities, and differences in processing plant compounds by insects are of course not surprising; they are already well documented in other insects, predominantly in butterflies and moths (Brower, 1970; Bowers, 1992; Rowell-Rahier and Pasteels, 1992; Hartmann and Witte, 1994). In *Oreina* species, the full description of allopatric and sympatric variations in host-derived defense requires further analysis of individual beetles freshly collected in the field. The real challenge, however, will be to evaluate the consequences for beetle fitness of this diversity and flexibility of defense.

Acknowledgments—This work was supported by grants of the Belgian Fund for Joint Basic Research and the Communauté Française de Belgique (ARC 93-3318) to J.M.P., of the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie to T.H., and of the Schweizerischer Nationalfonds to M.R.R. We thank Cl. Theuring for skillful technical assistence, L. Witte for GC-MS analyses, F. Eggenberger and B. Hägele for HPLC analyses, and H. Rowell for improving the English.

REFERENCES

Bourdonneé, J.-C., and Doguet, S. 1991. Données sur la biosystématique des Chrysolinina L.S. (Coleoptera: Chrysomelidae: Chrysomelinae). *Ann. Soc. Entomol. Fr.* 27:24–69.

Bowers, M.D. 1992. The evolution of unpalatability and the cost of chemical defense in insects, pp. 216–244, *in* B.D. Roitberg and M.B. Isman (eds.). Insect Chemical Ecology. An Evolutionary Approach. Chapman & Hall, New York.

BROWER, L.P. 1970. Plant poisons in a terrestrial food chain with implications for mimicry theory. *Proc. Annu. Biol. Colloq. Oregon State Univ.* 29:69–82.

DOBLER, S., and ROWELL-RAHIER, M. 1994a. Production of cardenolides versus sequestration of pyrrolizidine alkaloids in larvae of *Oreina* species (Coleoptera, Chrysomelidae). *J. Chem. Ecol.* 20:555–568.

DOBLER, S., and ROWELL-RAHIER, M. 1994b. Response of a leaf beetle to two food plants, only one of which provides a sequestrable defensive chemical. *Oecologia* 97:271–277.

EGGENBERGER, F., and ROWELL-RAHIER, M. 1991. Chemical defense and genetic variation. Interpopulational study of *Oreina gloriosa* (Coleoptera: Chrysomelidae). *Naturwissenschaften* 78:317–320.

EGGENBERGER, F., and ROWELL-RAHIER, M. 1992. Genetic component of variation in chemical defense of *Oreina gloriosa* (Chrysomelidae: Coleoptera). *J. Chem. Ecol.* 18:1375–1387.

EGGENBERGER, F., and ROWELL-RAHIER, M. 1993. Physiological sources of variation in chemical defense of *Oreina gloriosa* (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* 19:395–410.

EGGENBERGER, F., DALOZE, D., PASTEELS, J.M., and ROWELL-RAHIER, M. 1992. Identification and seasonal quantification of defensive secretion components of *Oreina gloriosa* (Coleoptera: Chrysomelidae). *Experientia* 48:1173–1179.

EHMKE, A., ROWELL-RAHIER, M., PASTEELS, J.M., and HARTMANN, T. 1991. Sequestration of ingested [^{14}C]senecionine N-oxide in the exocrine defensive secretions of chrysomelid beetles. *J. Chem. Ecol.* 17:2367–2379.

FREUDE, H., HARDE, K.W., and LOHSE, G.A. 1966. Die Käfer Mitteleuropas, Vol. 9. Goecke & Evers Verlag, Krefeld, Germany.

HARTMANN, T., and WITTE, L. 1995. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids, pp. 155–233, *in* S.W. Pelletier (ed.). Alkaloids: Chemical and Biological Perspectives, Vol. 9, Pergamon Press, Oxford.

Kühnelt, W. 1984. Monographie der Blattkäfergattung *Chrysochloa* (Coleoptera, Chrysomelidae), pp. 171–287, *in* Sitzung der mathem.-naturw. Klasse am 12 Oktober 1984, Springer-Verlag, Vienna.

PASTEELS, J.M., BRAEKMAN, J.-C., and DALOZE, D. 1988a. Chemical defense in the Chrysomelidae, pp. 233–252, *in* P. Jolivet, E. Petitpierre, and T. H. Hsiao (eds.). Biology of the Chrysomelidae. Kluwer, Dordrecht, Netherlands.

PASTEELS, J.M., ROWELL-RAHIER, M., RANDOUX, T., BRAEKMAN, J.-C., and DALOZE, D. 1988b. Pyrrolizidine alkaloids of probable host-plant origin in the pronotal and elytral secretion of the leaf beetle *Oreina cacaliae*. *Entomol. Exp. Appl.* 49:55–58.

PASTEELS, J.M., EHMKE, A., EGGENBERGER, F., ROWELL-RAHIER, M., and HARTMANN, T. 1992. Chemical defense in chrysomelid leaf beetles: storage of host-derived pyrrolizidine alkaloids versus de novo synthesized cardenolides. *Naturwissenschaften* 79:521–523.

PASTEELS, J.M., ROWELL-RAHIER, M., BRAEKMAN, J.-C., and DALOZE, D. 1994. Chemical defense in chrysomelid leaf beetles updated, pp. 289–301, *in* P.H. Jolivet, M.L. Cox and E. Petipierre (eds.). Novel Aspects of the Biology of Chrysomelidae. Kluwer, Dordrecht, Netherlands.

ROWELL-RAHIER, M. 1992. Genetic structure of leaf-beetle populations: Microgeographic and sexual differentiation in *Oreina cacaliae* and *O. speciosissima*. *Entomol. Exp. Appl.* 65:247–257.

ROWELL-RAHIER, M., and PASTEELS, J.M. 1992. Third trophic level influences of plant allelochemicals, pp. 243–277, *in* G.A. Rosenthal and M.R. Berenbaum (eds.). Herbivores. Their Interactions with Secondary Plant Metabolites, 2nd ed., Vol. II. Ecological and Evolutionary Processes. Academic Press, San Diego.

ROWELL-RAHIER, M., and PASTEELS, J.M. 1994. A comparison between allozyme data and phenotypic distances from defensive secretion in *Oreina* leaf-beetles (Chrysomelinae). *J. Evol. Biol.* 7:489–500.

ROWELL-RAHIER, M., WITTE, L., EHMKE, A., HARTMANN, T., and PASTEELS, J.M. 1991. Sequestration of plant pyrrolizidine alkaloids by chrysomelid beetles and selective transfer into the defensive secretions. *Chemoecology* 2:41–48.

ROWELL-RAHIER, M., PASTEELS, J.M., ALONSO-MEJIA, A., and BROWER, L.P. 1995. Relative unpalatability of leaf-beetles with either biosynthesized or sequestered chemical defense. *Anim. Behav.* 49:709–714.

VAN OYCKE, S., RANDOUX, T., BRAEKMAN, J.-C., DALOZE, D., and PASTEELS, J.M. 1988. New cardenolide glycosides from the defense glands of Chrysosilina beetles (Coleoptera: Chrysomelidae). *Bull. Soc. Chim. Belg.* 97:297–311.

WITTE;, L., RUBIOLO, P., BICCHI, C., and HARTMANN, T. 1993. Comparative analysis of pyrrolizidine alkaloids from natural sources by gas chromatography-mass spectrometry. *Phytochemistry* 32:187–196.