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Chemical defence in chrysomelid eggs and neonate larvae

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ABSTRACT. Eggs and neonate larvae of chrysomelid beetles (sub-tribes Chrysomelina and Phyllodectina) were investigated for the presence of defensive substances.

The two isoxazolinone glucosides (compounds 1 and 2), characteristic of the adult defence secretion, were detected in the eggs of all studied species. Compound 2, containing a nitropropionate, is always present in concentrations (above 10^{-2} M), which are highly deterrent to the ant *Myrmica rubra*. This compound is not at all or only slightly toxic to ants at 10^{-2} M. Compound 1, devoid of nitropropionate, is a minor constituent, and is neither deterrent nor toxic to ants.

The five *Chrysomela* species studied and *Phratora vitellinae* also sequester salicin in their eggs in amounts highly deterrent and toxic to ants. A single *Chrysomela* egg often contains enough salicin to kill an ant. While the isoxazolinones are discarded with the egg shells, salicin is used by neonate larvae as a precursor for the production of salicylaldehyde in the thoracic defence glands, already functional at hatching. No salicin could be detected in the eggs of those species whose larvae produce cyclopentanoid monoterpenes, even if they feed on Salicaceae. No larva of any species seems to be able to produce detectable amounts of monoterpenes at birth. A very early defence, possible only in those species using salicin as the precursor for their defensive secretion, could be highly advantageous in protecting the clustered larvae during the long process of hatching and in avoiding cannibalism between siblings.

Only trace amounts of oleic acid were found in the eggs of *Gastrophysa viridula*, in contrast to previous reports on its presence in large quantities in the American *G.cyanea*.

Key words. Chemical defence, insect–host plant interactions, Chrysomelidae, Chrysomelina, Phyllodectina, *Salix*, *Populus*, isoxazolinone glucosides, salicin, salicylaldehyde, cyclopentanoid monoterpenes, toxicity, feeding deterrency. *Myrmica rubra*.

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Introduction

Considerable advances have been made recently in the study of the defensive chemistry of chrysomelid larvae and adults. The identified allomones belong to different structural classes which match the taxonomic position of the insect secreting them. Apparent discrepancies can be explained by the use of host plant secondary compounds as precursors for the defensive allomones (Pasteels *et al.*, 1982, 1984). Within the sub-tribes Chrysomelina and Phyllodectina, adults secrete isoxazolinone glucosides 1 and 2 (Fig. 1) from their pronotal and elytral defence glands, whereas larvae secrete either monoterpenes or aromatic compounds from nine pairs of thoracic and abdominal glands. At least some aromatic compounds are derived from plant precursors, the best-documented example is the synthesis of salicylaldehyde from salicin in several insects feeding on *Salix* and *Populus* (Pasteels *et al.*, 1983).

The eggs, often brightly coloured, are laid in clusters on the foliage and are thus highly exposed to predation. Indeed the eggs of *Plagiodera versicolora* have a survival of only 25% in the field, and almost 50% of the clusters are completely destroyed. Observed enemies include migratory warblers, hemipteran nymphs, coccinelid adults and larvae, syrphid fly larvae, and egg parasitoids (M. J. Wade & F. Breden, personal communication). There might

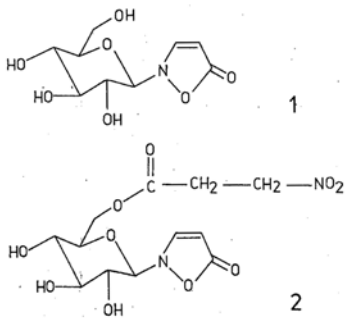

FIG. 1. Chemical structures of isoxazolinone glucosides 1 and 2 present in the adult defensive secretion and in the eggs of the species belonging to the sub-tribe Chrysomelina and the genus *Phratora*.

therefore be a strong selective pressure for the eggs to be chemically protected. Little is known, however, about the presence of toxins or repellents in chrysomelid eggs. Cardenolides have been reported in the eggs of *Chrysolina polita* and *C. coerulans* (Chrysolinina) (Pasteels & Daloze, 1977; Daloze & Pasteels, 1979). Oleic acid, which repels ants, has been reported in the eggs of *Gastrophysa cyanea* (Howard *et al.*, 1982).

We have investigated the presence of defensive allomones in the eggs of the Chrysomelina and Phyllodectina and followed their fate in neonate larvae in the hope of answering the following questions: Are the isoxazolinone glucosides, secreted by the adult defensive glands, also present in the eggs? Is salicin sequestered in the eggs of species feeding on Salicaceae? This is suggested by the fact that some neonate larvae seem to produce salicylaldehyde before feeding. Is oleic acid, found in large quantity in the eggs of the North American *G.cyanea* (40 μg per egg; Howard *et al.*, 1982), also found in the European *G.viridula*? When are egg allomones utilized further by the neonate larvae, and when are they abandoned with the egg shells? Do the neonate larvae start to produce autogenous defensive chemicals before feeding?

Additionally, toxicity and feeding deterrency of the egg allomones has been tested on the ant *Myrmica rubra*. Many ants are oophages (Du Merle *et al.*, 1978) and *M.rubra* foragers were often seen exploring the foliage of the host plants of various chrysomelids. *M.rubra* thus could exemplify generalized potential predators.

Materials and Methods

The insects

Various chrysomelid species, listed in Table 1, were collected in the field in Belgium, except for *Chrysomela saliceti* collected in the neighbourhood of Frankfurt (Germany). They were bred in the laboratory on the food plants listed in Table 2. Egg volumes were estimated from the linear dimension of the eggs, making the assumption that they were ellipsoidal. Hexane extracts of neonate larvae were made from larvae collected at hatching from egg clusters isolated from the food plant. For *G.cyanea*, separate extracts were made from the gluey

yellow secretion sticking to the egg shell and collected on filter papers, from the fluid content of the eggs after puncture with a needle, and from the remains of the eggs after hatching.

Chemical analysis

Eggs and neonate larvae of all the species studied were extracted three times with CH_2Cl_2:CH_3OH (1:1 v/v). The combined organic extracts were evaporated under vacuum. The quantitative analysis of isoxazolinone glucosides was performed by photodensitometry using TLC plates (Silicagel G, F254, Merck), developed with $CHCl_3$:CH_3OH (8:2 v/v) and visualized with UV light at 260 nm. For each egg extract, three solutions of different concentrations were prepared by adding known volumes of propanol. Duplicate analysis of 2 μl of each of these samples were performed on a TLC plate. Solutions of isoxazolinone 2, also at three different concentrations, were used as an internal standard. Absorbances were measured quantitatively using a Shimadzu Type 920 high-speed TLC scanner.

The amounts of isoxazolinones present in the samples were estimated from a calibration curve determined for isoxazolinone 2, which was found to be linear over the concentration range used. Quantities given in Table 1 are mean values, obtained by averaging the six measurements (duplicated analysis for three dilutions) on each extract.

Salicin was quantitatively determined by HPLC, following the method of Sticher *et al.* (1981). Identification of oleic acid from *G.viridula* eggs was performed by extraction with CH_2Cl_2, followed by methylation with CH_2N_2 and quantitative GC analysis using an authentic sample of methyl oleate as standard.

The major lipids present in *G.viridula* eggs were identified as triglycerides by mass spectrometry, after flash chromatography (eluent: benzene:hexane 8:2 v/v).

The presence of salicylaldehyde and monoterpenes in neonate larvae was investigated by GC (Hewlett-Packard 402, Carbowax 20M column, 1.80 m, programme from 150 to 200°C).

Feeding deterrency

Groups of fifty ants, foragers of *M.rubra* taken from laboratory cultures, were isolated in closed Petri dishes (40×2 cm). The ants were starved for 16 h before the experiments, but water was supplied. During the test the water supply was removed and the ants given the choice between either 50 μl of pure sucrose (10^{-1} M) or 50 μl of the same sucrose solution in which various amounts of the tested substances were dissolved. These test liquids were placed in 7 mm depressions made in a paraffin surface on a glass slide (Fig. 2A). After 5 min the number of ants feeding on each solution was counted. A delay of 5 min was necessary for the ants to recover from the disturbance and to make a choice, after which their distribution remains stable. Twelve replicates, each with new ants, were made with each substance tested and the results were analysed statistically by using the Wilcoxon matched-pairs signed-ranks test (Siegel, 1956). The relative position of the two solutions were alternated between repetitions (Fig. 2B).

Toxicity tests

Three samples of ants were isolated as for the deterrency tests, but this time were deprived of both food and water for 16 h. Then each group was given either pure water, or a sugar solution (10^{-1} M sucrose), or a 10^{-2} M solution of the test compound in 10^{-1} M sucrose. Drops of 5 μl were given in succession until the ants were satiated. In this way, the ants drank on average up to 1 μl per day. As much as possible, the ants received the same amount of liquid in all the three groups. In no case did the ants receive less of the test solution than the amount of pure water drunk by the control group. Mortality was recorded every morning.

Results

Occurrence of isoxazolinones in the eggs (Table 1)

Both isoxazolinones (1 and 2) were detected in the eggs of all species studied. They were not detected in the yellow gluey secretion which cover the eggs of *G.viridula*, but were found in the fluid content of the eggs of this latter species.

Quantitative variations in absolute content were observed between the different species, and seem to be due in large part to differences in egg sizes. Indeed, the concentrations found in the eggs are remarkably similar (between 2 and

TABLE 1. Total amounts (mean ±SE) and concentrations of isoxazolinones 1 and 2 in the eggs of species belonging to the sub-tribes Chrysomelina and Phyllodectina.

	No. of eggs	μg/egg	Concentrations (M)
Chrysomelina			
Ch.populi L.	149	8.1±0.3	4.37×10^{-2}
Ch.populi L.	52	5.8±0.3	3.14×10^{-2}
Ch.tremulae F.	154	5.5±0.3	3.25×10^{-2}
Ch.saliceti Weise	75	++	
Ch.20-punctata Scop.	215	++	
G.viridula De Geer	232	1.2±0.2	2.00×10^{-2}
Pl.versicolora Laich.	230	++	
Phyllodectina			
Ph.vitellinae L.	237	++	
Ph.laticollis Suffr.	261	++	
Ph.tibialis Suffr.	226	++	

++: No quantitative determination.

4×10^{-2} M). The amounts reported are the sum of both glucosides. However, 2 was always the major compound. In those species for which no quantitative data are given, TLC analyses suggested that the two compounds are present in concentrations similar to those found in the other species.

Occurrence of salicin in the eggs (Table 2)

The species of insects living on host plants other than members of Salicaceae naturally do not contain salicin in their eggs. However, the eggs of only some of the insects feeding on salicaceous plants contain salicin, and those species are also those whose larvae produce salicylaldehyde. No salicin was detected in the eggs of *Plagiodera versicolora*, *Phratora laticollis* and *Ph.tibialis*, all of which feed on *Salix* and *Populus*, but have larvae which produce exclusively cyclopentanoid monoterpenes.

Large differences in the amounts of salicin present were observed between species, which cannot be accounted for simply by differences in egg volumes. The small amount detected in the eggs of *Ph.vitellinae* is due in part to their small size, but possibly also to the fact that they were not cultured on their preferred host-plant, *Salix nigricans* (Rowell-Rahier, 1984a, b).

Occurrence of oleic acid in the eggs of G.viridula

Only trace amounts of free oleic acid were found in the eggs of *G.viridula*. At best, only 0.065 μg/egg was detected. The major lipid fraction is composed of a mixture of triglycerides

TABLE 2. Amounts (average from more than 200 pooled eggs) and concentrations of salicin in the eggs of species belonging to the sub-tribes Chrysomelina and Phyllodectina.

	Host-plant	μg/egg	Concentration (M)
Chrysomelina			
*Ch.populi**	*P.trichocarpa*	14.0	1.0×10^{-1}
*Ch.populi**	*P.trichocarpa*	6.0	4.6×10^{-2}
*Ch.tremulae**	*P.trichocarpa*	3.0	2.5×10^{-2}
*Ch.saliceti**	*S.purpurea*	5.0	
*Ch.20-punctata**	*S.purpurea*	2.1	
G.viridula	*R.obtusifolium*	0	0
Pl.versicolora	*S.fragilis*	0	0
Phyllodectina			
*Ph.vitellinae**	*P.trichocarpa*	0.3	8.4×10^{-3}
Ph.laticollis	*P.trichocarpa*	0	0
Ph.tibialis	*S.purpurea*	0	0

* Species whose larvae produce salicylaldehyde from their defensive glands.

containing saturated and unsaturated C_{16} and C_{18} fatty acids.

Defensive allomones in neonate larvae

Only the thoracic glands are functional at hatching, and all neonate larvae are able to extrude them when disturbed while still attached to the egg shell. Only the *Chrysomela* species produce a visible secretion smelling of salicylaldehyde. No secretion was observed in those larvae which later produce cyclopentanoid monoterpenes.

The presence of salicylaldehyde was confirmed in the extract of neonate larvae of *Ch.20-punctata* and *Ch.saliceti*, and also in much smaller amount in the extract of *Ph.vitellinae*. No monoterpenes could be detected in the extracts of neonate larvae of any of the species studied (i.e. *G.viridula*, *Pl.versicolora*, *Ph.vitellinae*, *Ph.tibialis*, *Ph.vulgatissima*), and none of them seems able to produce those from birth.

Neither salicin nor the isoxazolinones were detected in neonate larvae. Thus most if not all of the salicin originally present in the eggs must have been transformed into salicylaldehyde. In *G.viridula*, isoxazolinones were detected in the extract of the egg shells left after hatching. These must be derived from either the embryo or the larval stage which develops within the egg shell before hatching (Renner, 1970).

Deterrent activity of salicin and the isoxazolinones (Table 3)

Isoxazolinone glucoside 1 proved to be only slightly deterrent to ants at a concentration (3×10^{-2} M) well above its concentration in the eggs. This compound is far less abundant in the eggs than its nitroproprionate derivative (2).

Both salicin and isoxazolinone glucoside 2 are far more deterrent to ants, being still slightly active at 1×10^{-3} M. They were strongly deterrent at the concentrations observed in the eggs. The behaviour of the ants, however, was quite different when they tasted the solutions of salicin or of 2 at these concentrations. The ants tried the solutions of salicin, but fed only for a short time and then left the solution without any signs of discomfort. A few ants were thus always feeding (Fig. 2B). In contrast, the ants in contact with solutions of 2 immediately retreated, dragging their mouth parts and antennae on the substrate (Fig. 2A).

Toxicity of salicin and the isoxazolinones for ants (Fig. 3)

Small drops (5 μl) of 10^{-2} M solutions of salicin and isoxazolinone 2 were accepted by water-deprived ants, when no choice was available, even though these solutions proved to be deterrent in the less severe conditions of the preceding experiments.

Salicin proved to be highly toxic. 50% mortality was reached after 2 days of the experimental regime, during which the ants drank on average 1.7 μl/ant. In the control groups the consumption was during the same period, respectively 1.7 μl/ant of pure water, and 1.9 μl/ant of sucrose solution. The LD_{50} may be estimated as 5 μg of salicin/ant in 2 days; this is the amount found on average in one *Chrysomela* egg (see Table 2).

A 10^{-2} M solution of isoxazolinone 2 in 10^{-1} M sucrose seems to be only slightly toxic at best. Mortality was about the same as in the starved group receiving only water, and somewhat higher than in the group which received the sucrose solution (Fig. 3B). At the end of the experiment the ants had eaten on average 19 μg

TABLE 3. Percentages of ants (*M.rubra*) feeding on a concentration range of egg allomones solutions. See text for further details.

Egg allomones	Concentration tested (M/l in sucrose 10^{-1} M)					
	10^{-1}	3×10^{-2}	10^{-2}	3×10^{-3}	10^{-3}	3×10^{-4}
Salicin	12*	26*	23*	37*	38*	45
Isoxazolinone 1	NT	39*	51	NT	NT	NT
Isoxazolinone 2	NT	0*	0*	31*	37*	NT

NT: concentration not tested.
* $P<0.05$ in Wilcoxon matched-pairs signed-ranks test.

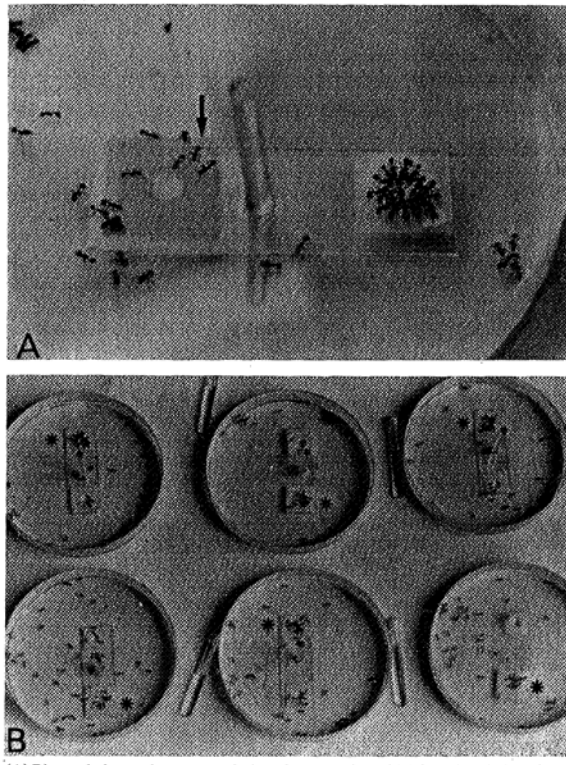

FIG. 2. (A) Binary choice test between a solution of sucrose 10^{-1} M (right), and a solution of isoxazolinone 2 10^{-2} M in sucrose 10^{-1} M. (B) Illustration of six replications of binary choice tests between sucrose 10^{-1} M (marked by *) and solutions of salicin 10^{-2} M in sucrose 10^{-1} M.

of isoxazolinone 2/ant in 8 days; this is the equivalent of several chrysomelid eggs. Isoxazolinone 1 was not toxic at all after 5 days. The experiment had to be interrupted because the supply of substance was exhausted; 10.2 μg of 1 per ant were consumed during this period.

Discussion

Defence mechanisms of insect eggs has been reviewed by Hinton (1981) and, as in other developmental stages, they include spectacular forms of crypsis or of chemical defence associated with bright colours and clustering.

The eggs of several species of Lepidoptera are covered with toxic larval setae carried there by the females, and those of some Mantispidae (Neuroptera) are covered with poisonous fluid from the accessory glands (Hinton, 1981), but more often toxins are incorporated within the eggs themselves. These toxins or deterrents may have a dual origin. They can be sequestered from the host plant and incorporated in the eggs

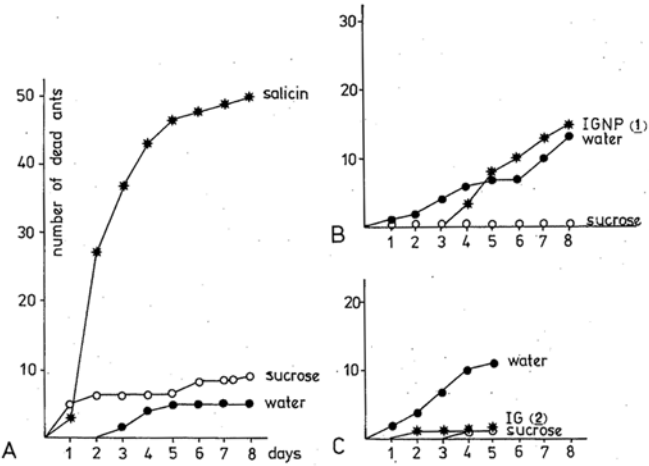

FIG. 3. Cumulative mortality curves for ants drinking a 10^{-2} M solution of salicin in sucrose 10^{-1} M (A), of isoxazolinone glucoside 2 in sucrose 10^{-1} M (B), of isoxazolinone glucoside 1 in sucrose 10^{-1} M (C), as well as for control groups drinking either pure water or a pure solution of sucrose 10^{-1} M.

as they are in other tissues, e.g. cardenolides in the eggs of the monarch butterfly (Reichstein *et al.*, 1968), the grasshopper *Poekilocerus bufonius* (von Euw *et al.*, 1967), or the bug *Oncopeltus fasciatus* (Duffey & Scudder, 1974). Other are synthesized by the females, e.g. the alkaloid coccinellin in *Coccinella septempunctata* (Pasteels *et al.*, 1973), cardiac glycosides in *Chrysolina* species (Pasteels & Daloze, 1977; Daloze & Pasteels, 1979), or cyanogenic glycosides in *Zygaena* species (Davis & Nahrstedt, 1982).

Our results show that in some chrysomelid species females protect their eggs by two methods. All species incorporate in their eggs isoxazolinones, most probably biosynthesized *de novo*. Further, in some species salicin is sequestered from their food and transferred to their eggs.

The isoxazolinone glucosides 1 and 2, characteristic of the adult defensive secretion, were found in the eggs of all studied species of Chrysomelina and Phyllodectina. The occurrence of identical compounds in the eggs and in adult defensive glands was already reported in the species of Chrysolinina producing cardenolides, and could represent a general feature of the members of Chrysomelinae.

The strong deterrent activity of isoxazolinone 2 against ants at concentrations found in the eggs suggests that it has a protective role against predators. Moreover, its location in the fluid content of the egg might be particularly efficient to deter generalist predaceous insects like Heteroptera and the nymphs of Neuroptera (Jolivet, 1950), which puncture the eggs with their mouth parts and suck the fluid content; the gluey secretion covering the egg could provide an initial mechanical barrier. Isoxazolinone 2 was found to have no or little toxicity to ants at 10^{-2} M. However, the concentrations found in the eggs are somewhat higher and 3-nitropropionic acid which can be easily released from 2 by hydrolysis, is known to be highly toxic in vertebrates and invertebrates (Hutchins *et al.*, 1984; Bell, 1974; Shenk *et al.*, 1976). Further experiments with different potential predators are necessary to assess the protective role of 2 for the eggs. In contrast to 2, isoxazolinone 1 was found to be neither highly toxic nor deterrent, and its biological significance remains obscure. Being always present in much smaller concentration

than 2, both in the eggs and in the adult secretion, it may just represent an inactive precursor of 2.

The incorporation of salicin in the eggs is certainly highly beneficial for the insects, and has evolved at least twice in the Chrysomelinae, once by a *Chrysomela* and once by *Phratora vitellinae*. An anthocorid bug fed avidly on eggs of *Ph. vitellinae* but refused those of *Ch. tremulae* (M. Rowell-Rahier, personal observation) which are 10 times richer in salicin. Not only is salicin deterrent and highly toxic for ants, and possibly to other predators, but it also allows the neonate larvae to be efficiently defended at a very vulnerable stage (see below for discussion of defence of neonate larvae). Among those species feeding on Salicaceae, only the species in which the larvae produce salicylaldehyde incorporate salicin in their eggs, although salicin would insure protection of the eggs in other species also. The incorporation of salicin in the eggs by the adult females and its use by the larvae as precursor for defensive allomones both need as a first step the sequestration of the plant toxin which must depend on the same physiological adaptation.

Salicin is highly deterrent against *M. rubra* at the concentration found in the eggs and also toxic to that ant in the amounts found in one single egg of most *Chrysomela* species. The values reported here on the deterrent activity of salicin for *M. rubra* are not in agreement with previous reports on its activity (Pasteels *et al.*, 1983). In the present tests salicin was found to be strongly deterrent at 10^{-2} M and slight but still significant deterrency was observed at 10^{-3} M, whereas in the previous experiments no deterrent activity could be found at 10^{-2} M. This simply reflects that the results of such tests are strongly dependent on the methodology used. The activity of different compounds can only be compared if the tests are done under exactly the same conditions. In the previous experiments the tests were made on entire ant societies, and the consumption of the solutions was measured by weight. This method proved to be far less sensitive than the one adopted in the present study for the following reasons. First, the physiological state of whole nests is far less easy to standardize than that of fifty isolated foragers. Second, the test had to be done with 'extremely thirsty' ants, so that the loss of weight due to the actual consumption of solution would be higher than the loss due to evaporation. Third, the quantification of consumption by weight was inaccurate because the ants sometimes dropped various objects in the solutions or they carried part of it away when they dragged their mouthparts and antennae on the substrate. We found the new method described in this paper much more reliable and repeatable (Fig. 3B), especially for small deterrent activities.

Our results show that the isoxazolinones are discarded by the neonate larvae which rely on volatile compounds for their defence. The salicin present in the eggs, however, is transformed into salicylaldehyde, providing a much earlier defence than if the larvae had to isolate it from their food. A strong defence in the neonate is probably a significant advantage because it can protect the larvae both against predation and against cannibalism. Cannibalism is not rare amongst herbivorous larvae hatching from egg clusters (Polis, 1981). It has been reported for *Plagiodera versicolora* and can account for a loss of 45% of the hatching larvae (M. J. Wade & F. Breden, personal communication). Hatching is a prolonged process, during which the larvae remain for extended periods attached to the egg shells, with only the head and the thorax protruding. After hatching, the neonate larvae usually rest for sometime on the shells. These clusters of larvae would be vulnerable to predators unless they were protected by thoracic secretion. If this is so, the clustering will increase the efficiency of defence by the pooling of the individual secretions, which are still limited at this stage (Raupp, 1982; Tostowaryk, 1972). Paradoxically, only those larvae which depend on salicin, normally found in their food, seem to be able to produce a very early defensive secretion before feeding, because of provisioning by their mother. The biosynthesis of cyclopentanoid monoterpenes is a heavier metabolic burden for the larvae than is the transformation of salicin into salicylaldehyde (Rowell-Rahier & Pasteels, 1986). Comparative studies of survival of eggs and neonate larvae with different defensive strategies are planned.

Only trace amounts of oleic acid were found in the eggs of *G. viridula*: hence this compound is not likely to be involved in defence of the eggs. Howard *et al.* (1982) suggested that oleic acid could not be the sole defensive agent of the eggs of *G. cyanea*. Because of the extraction techniques they used, polar compounds like the isox-

azolinones could not be detected; it would be worthwhile to look for their presence in this species.

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