Chemical defence in chrysomelid eggs and neonate larvae

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

The two isoxazolizone glucosides (compounds 1 and 2), characteristic of the adult defence secretion, were detected in the eggs of all studied species. Compound 2, containing a nitropropanolate, 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 nitropropanolate, 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 isoxazolizones 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, Chrysolina, Phyllocoelina, Salix, Populus, isoxazolizone glucosides, salicin, salicylaldehyde, cyclopentanoid monoterpenes, toxicity, feeding deterrent. 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 (Pastewka et al., 1982, 1984). Within the sub-tribes Chrysolinae and Phyllocoelinae, adults secrete isocladolone glucosides 1 and 2 (Fig. 1) from their pronotal and elytral defence glands, whereas larvae secrete either mono- or sesquiterpenoid 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 (Pastewka 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 23% in the field, and almost 50% of the clusters are completely destroyed. Observed enemies include migratory warblers, hemipteran nymphs, coccinellid adults and larvae, syrphid fly larvae, and egg parasitoids (M. J. Wade & F. Brooker, personal communication). There might 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 Chrysomela popina and C. coerulea (Chrysolinae) (Pastewka & Dalzen, 1977; Dalzen & Pastewka, 1979). Oleic acid, which repels ants, has been reported in the eggs of Gastrophyes cyanus (Howard et al., 1982).

We have investigated the presence of defensive allomones in the eggs of the Chrysolinae and Phyllobotrinae and followed their fate in neonate larvae in the hope of answering the following questions: Are the isocladolone 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. cyanus (5 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 shell? Do the neonate larvae start to produce autogenous defensive chemicals before feeding?

Additionally, toxicity and feeding deterrence of the egg allomones has been tested on the ant Myrmica rubra. Many ants are coccinellid enemies (Du Mortel 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 saltatoria 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. cyanus, 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 analyses**

Eggs and neonate larvae of all the species studied were extracted three times with CHCl₃:CH₃OH (1:1 v/v). The combined organic extracts were evaporated under vacuum. The quantitative analysis of isoxazolino glucosides was performed by photodensitometry using TLC plates (Silica gel G, P254, Merck), developed with CHCl₃:CH₃OH (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 propaeno propaeno. Duplicate analysis of 2 μl of each of these samples were performed on a TLC plate. Solutions of isoxazolino 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 isoxazolino glucosides present in the samples were estimated from a calibration curve determined for isoxazolino 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. Salvin was quantitatively determined by HPLC, following the method of Stierer et al. (1981). Identification of oleic acid from G. viridula eggs was performed by extraction with CHCl₃, followed by methylation with CH₃N 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 salicylddehyde and monoterpenes in neonate larvae was investigated by GC (Hewlett-Packard 492, Carbowax 20M column, 1.80 m, programme from 150 to 200°C).

**Feeding deterrents**

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 (10⁻⁴ 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 min 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 deterrentness tests, but this time they were deprived of both food and water for 16 h. Then each group was given either pure water, or a sugar solution (10⁻⁴ M sucrose), or a 10⁻⁴ M solution of the test compound in 10⁻⁴ 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 groups. Mortality was recorded every morning.

**Results**

**Occurrence of isoxazolino glucosides in the eggs**

(Table 1)

Both isoxazolino glucosides (1 and 2) were detected in the eggs of all species studied. They were not detected in the yellow gummy 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
The amounts reported are the sum of both glucosides. However, 2 was always the major compound. In these 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, Platanus laticollis and Ph. abiusis, 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. stellineae 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 nigra (Roser-Zahler, 1984a, b).
containing saturated and unsaturated \( \text{C}_{16} \) and \( \text{C}_{18} \) fatty acids.

**Defensive allelochemicals 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 *Chrysonota* species produce a visible secretion smelling of salicylaldehyde. No secretion was observed in these larvae which later produce cyclopentanoid monoterpenes.

The presence of salicylaldehyde was confirmed in the extract of neonate larvae of *Ch. japonica* 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 other species studied (i.e. *G. viridula*, *P. versicolora*, *Ph. vitellinae*, *Ph. tibialis*, *Ph. volgatissima*), and none of them seem able to produce those from birth.

Neither salicin nor the isoxazoliones 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*, isoxazoliones 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 (Reznor, 1970).

**Deterrent activity of salicin and the isoxazoliones (Table 3)**

Isoxazolione glucoide 1 proved to be only slightly deterrent to ants at a concentration (3\( \times 10^{-3} \) mg/ml) well above its concentration in the eggs. This compound is far less abundant in the eggs than its nitropropionate derivative (2).

Both salicin and isoxazolione glucoide 2 are far more deterrent to ants, being still slightly active at 1\( \times 10^{-3} \) mg/ml. 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 isoxazoliones for ants (Fig. 3)**

Small drops (5 \( \mu \)l) of 10\(^{-3}\) mg solutions of salicin and isoxazolione 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 experiment.

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 \( \mu \)l/ant. In the control groups the consumption was during the same period, respectively 1.7 \( \mu \)l/ant of pure water, and 1.9 \( \mu \)l/ant of sucrose solution. The LD\(_{50}\) may be estimated as 5 mg of salicin/ant in 2 days; this is the amount found on average in one *Chrysonota* egg (see Table 2).

A 10\(^{-3}\) mg solution of isoxazolione 2 in 10\(^{-3}\) mg 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 \( \mu \)g

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<th>Egg allelochemes</th>
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<td>Salicin</td>
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<td>Isoxazolione 1</td>
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<td>Isoxazolione 2</td>
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<td>NT: concentration not tested.</td>
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* P<0.05 in Wilcoxon matched-pairs signed-ranks test.
FIG. 2. (A) Binary choice test between a solution of sucrose $10^{-3} \text{M}$ (right) and a solution of isoxazolone 2 $10^{-3} \text{M}$ in sucrose $10^{-1} \text{M}$. (B) Illustration of six replicates of binary choice tests between sucrose $10^{-3} \text{M}$ (marked by *) and solutions of solute $10^{-3} \text{M}$ in sucrose $10^{-1} \text{M}$.

of isoxazolone 2/ant in 8 days; this is the equivalent of several chrysomelid eggs. Isoxazolone 1 was not toxic at all after 8 days. The experiment had to be interrupted because the supply of substance was exhausted; 10.3 mg 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 egg itself. These toxins or deterrents may have a dual origin. They can be sequestered from the host plant and incorporated in the eggs.
as they are in other tissues, e.g. cardenolides in the eggs of the monarch butterfly (Reichstein et al., 1958), the grasshopper Poekilocerus buparius (van Emden et al., 1965), or the bag Oncopeltus fasciatus (Duffey & Scudder, 1974). Other are synthesized by the females, e.g. the alkaloid coecinolin in Cocinella septempunctata (Pasterk et al., 1973), cardiac glycosides in Chrysomela species (Pasterk & Dolez, 1977; Dolez & Pasterk, 1979), or cyanogenic glycosides in Zygaena species (Davis & Nahmstedt, 1982).

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

The isoaxazolino glucosides 1 and 2, characteristic of the adult defensive secretion, were found in the eggs of all studied species of Chrysoecina and Phytaloection. The occurrence of identical compounds in the eggs and in adult defensive glands was already reported in the species of Chrysoecina producing cardenolides, and could represent a general feature of the members of Chrysoecinae.

The strong deterrent activity of isoaxazolino 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 predators insects like Heteroptera and the nymphs of Neuroptera (Jolivet, 1950), which puncture the eggs with their mouth parts and suck the fluid content; the gummy secretion covering the egg could provide an initial mechanical barrier. Isoaxazolino 2 was found to have no or little toxicity to ants at 10−4 M. However, the concentrations found in the eggs are somewhat higher than the toxicity of carotenoids and cyanogenetic 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; Sheik 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, isoaxazolino 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 vitiulae. An anthropod bug fed avidly on eggs of Ph. willistoni but refused those of Ch. thebanae (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 larva 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 salicylalddehyde 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 a 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 in agreement with previous reports on its activity (Pateels et al., 1983). In the present tests salicin was found to be strongly deterrent at 10^{-3} M and slight but still significant deterrent was observed at 10^{-2} 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 thirty' ants, so that the loss of weight due to the actual consumption of salicin 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 isoxasoliones are discarded by the neonate larvae which rely on volatile compounds for their defence. The salicin present in the eggs, however, is transformed into salicylalddehyde, 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 (Polls, 1981). It has been reported for Plagiosternum verticidens and can account for a loss of 45% of the hatching larvae (M. J. Wade & F. Breeden, 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 would be sufficient for this stage (Raup, 1982; Tocotowsky, 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 cyclopropanoid monoterpenes is a heavier metabolic burden for the larvae than is the transformation of salicin into salicylalddehyde (Rowell-Rahier & Pateels, 1980). 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 defense of the eggs. Howard et al. (1982) suggested that oleic acid could not be the sole defensive agent of the eggs of G. cyanae. Because of the extraction techniques they used, polar compounds like the isox-
azoilones could not be detected; & would be worthwhile to look for their presence in this species.

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References