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## Evolution of exocrine chemical defense in leaf beetles (Coleoptera: Chrysomelidae)

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**Summary.** In this review we speculate on possible scenarios for the evolution of the very high diversity in chemical compounds liberated by exocrine glands of adults Chrysomelidae. Shift in host plant affinities and subsequent adaptation of the beetles to the plant toxins strongly influence the nature of the beetles' chemical defense.

**Key words.** Cardenolides; dipeptides; isoxazolinone glucoside; pyrrolizidine alkaloids; host plant influence; toxin sequestration.

The name 'Chrysomelidae' is derived from the Greek *chrysos*, gold, and *melolanthion*, beetle, referring to the bright metallic colors characteristic of many members of

this taxon. These colors are not there simply for the pleasure of amateur entomologists, but rather they have biological significance as intra- or interspecific signals. In

leaf beetles, unlike some other groups of insects (e.g. Lepidoptera, Orthoptera), no sexual dimorphism in coloration is known. Aposematism is probably the main 'raison d'être' for their colorful appearance. Indeed, the Chrysomelidae are well known as toxic insects, protected by a great diversity of chemicals.

The purpose of this review is to suggest possible scenarios for the evolution of diversity in chemical defense of Chrysomelidae. We will base our hypotheses on comparative morphological and chemical data, and on ecological factors, such as the influence of host plants on these herbivores. The toxins utilized for defense can be systemic<sup>12, 14</sup> or stored in specialized organs<sup>22</sup>. We will restrict our discussion to the defensive compounds secreted by exocrine glands of adult beetles. The evolution of chemical defense in the larvae<sup>21, 23, 25</sup> will not be discussed here.

#### Morphology and distribution of adult defensive glands

The structure and distribution of defensive glands on the body of leaf beetles are somewhat unusual for insects. Generally insect defensive glands possess a large cuticular reservoir, in which the secretion accumulates and where the last steps of the biosynthesis of toxins can occur (e.g. in chrysomelid larvae<sup>22</sup>). In adult leaf beetles the secretion accumulates not in a reservoir but in vacuoles of the secretory cells and in extracellular spaces. These cells converge on a linear duct which opens directly on the body surface (fig. 1). Another unusual feature is the fact that an individual possesses dozens of glands, whose openings are situated all over the pronotum and the elytra. The distribution of the openings varies between species, but the secretory activity is often greatest along the lateral margins of the body (fig. 2).

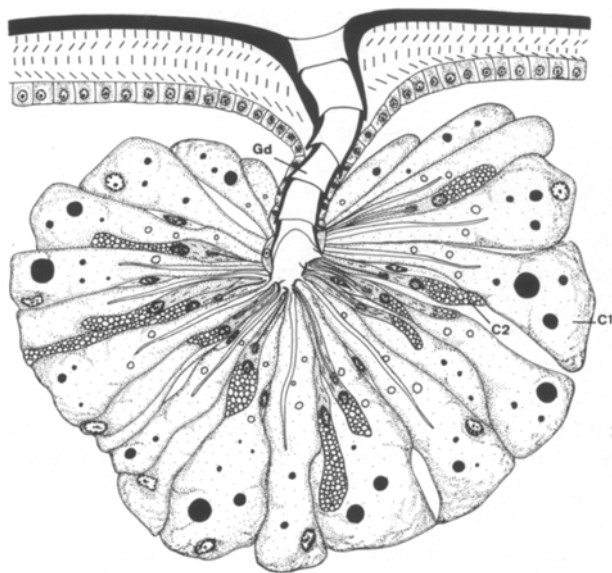


Figure 1. Defensive gland of *Leptinotarsa decemlineata*; C<sub>1</sub> and C<sub>2</sub>: gland cells; Gd: duct (from Deroe and Pasteels<sup>8</sup>).

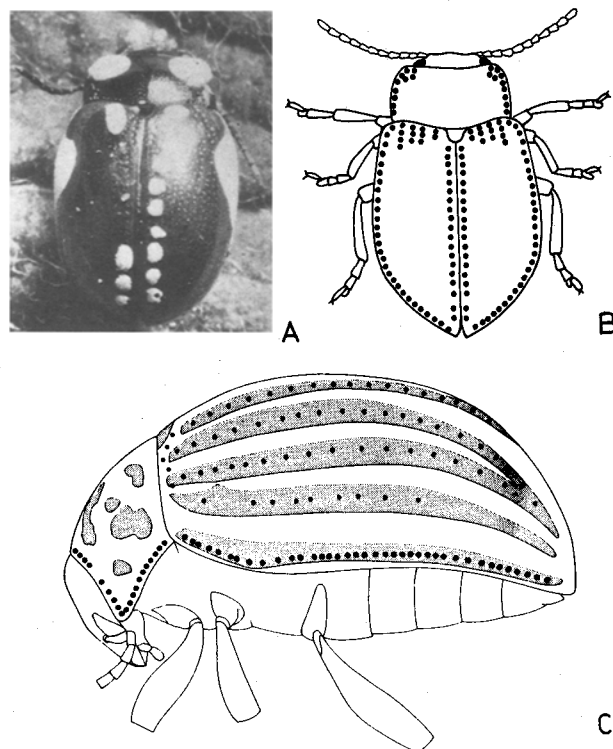


Figure 2. Distribution of defensive glands in leaf beetles. A Lyophilized *Chrysolina polita*. The dried secretion appears in white and is most abundant laterally. B, C Distribution of defensive glands in *C. polita* (B) and *Leptinotarsa decemlineata* (C). Each black circle represents one gland (B from Deroe and Pasteels<sup>9</sup>, C from Deroe and Pasteels<sup>8</sup>).

Defensive glands have been observed so far in the adults of 4 out of 19 chrysomelid subfamilies: in all Criocerinae and Chrysomelinae studied, and in some Alticinae and Galerucinae. Morphologically the glands are remarkably similar in these four subfamilies. Details of their ultra-

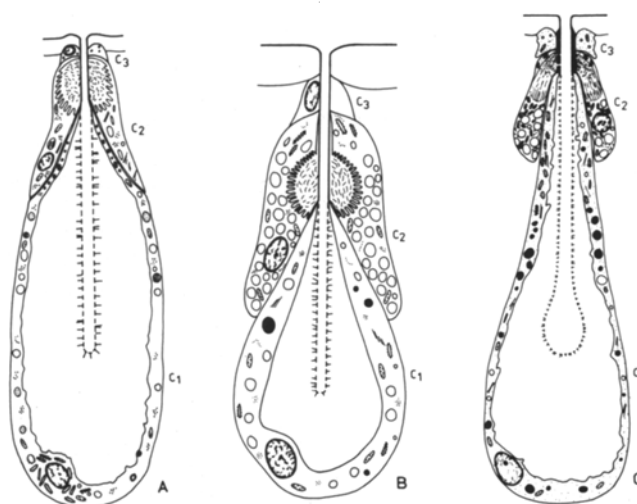


Figure 3. General organization of gland units in *Lilioceris lili* (Criocerinae) (A), *Galerucella tenella* (Galerucinae) (B), and *Gastrophysa viridula* (Chrysomelinae) (C). C<sub>1</sub> and C<sub>2</sub> gland cells, C<sub>3</sub> canal cell (redrawn from Deroe<sup>7</sup>).

structure are given in figure 3. A typical gland consists of several identical units which open independently into the duct. As is frequently observed in integumentary glands of insects, each unit is made of groups of three cells surrounding the common epicuticular ductule (Typ III glands<sup>17</sup>). Only the most apical cell of each series has no secretory function. Minor morphological differences are found between taxa in the relative proportion of the different cell types in each unit. The same general organization is observed in dermal glands which, in many insects, are made of single units scattered over the surface of the sclerites. In the course of evolution, dermal glands might have provided the precursor for chrysomelid defensive glands.

In the Galerucinae and the Alticinae the glands are often reduced or even absent, suggesting a secondary loss of this mode of defense, and are replaced by reflex bleeding and escape by jumping, respectively<sup>9</sup>.

Based on a study of wing morphology and male genitalia, the four subfamilies with glands form a monophyletic lineage<sup>15</sup> (Jolivet, pers. comm.). This view is supported by the structural similarity of the glands. Although affinities between the Alticinae and the Galerucinae are conventionally recognized, the Chrysomelinae and the Criocerinae are often placed in different lineages<sup>28</sup> (Cromson and Schmitt, pers. comm.). On the basis of their morphological similarity, we will consider the glands of the four subfamilies as homologous and as having a common origin.

Table 1. Distribution of adult defensive compounds in the Chrysomelinae and Criocerinae (Chrysomelidae, Coleoptera).

Beetles	Host plants	Secretions
<b>Subfamily Criocerinae</b>		
<i>Lilioceris lili</i>	Liliaceae	Phenylalanine derivatives
<b>Subfamily Chrysomelinae</b>		
1. Subtribe Doryphorina: <i>Leptinotarsa decemlineata</i>	Solanaceae	$\gamma$ -Glutamyl dipeptide
2. Subtribes Chrysomelina 6 genera, 9 spp.	Salicaceae, Betulaceae, Polyganaceae	Isoxazolinone and nitropropanoic acid glucosides
3. and Phyllodectina: 1 genus, 3 spp.		
4. Subtribe Chrysolinina*: 12 spp. of <i>Chrysolina</i>	Lamiaceae, Ranunculaceae, Plantaginaceae, Scrophulariaceae	Cardenolides
<i>Chrysolina dydimata</i>	Hypericaceae	1 cardenolide
4 other spp. of <i>Chrysolina</i>	Hypericaceae	6-Oxosteroids
7 spp. of <i>Oreina</i>	Apiaceae	Cardenolides
<i>O. speciosissima</i>	Asteraceae	Cardenolides and pyrrolizidine N-oxides
<i>Oreina cacaliae</i>	Asteraceae	Pyrrolizidine N-oxides

\* Ethanalamine is present in all secretions independently of the type of compound secreted.

### Comparative defensive chemistry

The chemical nature of the toxins present in the adult glands is well known in European species of the tribe Chrysomelini in the subfamily Chrysomelinae. Outside this group information is scanty.

The Chrysomelini exhibit a remarkably diverse defensive chemistry, including compounds from many different chemical classes (e.g. cardenolides, dipeptides, isoxazolinone glucosides and pyrrolizidine alkaloids) and of undoubtedly different biosynthetic origins. A list of the compounds identified and of the corresponding species is given in Pasteels et al.<sup>22</sup>, and additional new ones are reported in Pasteels et al.<sup>24</sup> and Van Oycke et al.<sup>31</sup>. The types of defensive compounds identified in the different taxa are summarized in table 1. We will not give more specific chemical details here. With few exceptions, there is a good correlation between the types of compounds secreted and the classification of the beetles at the subtribal level.

In the subtribe Doryphorina, the secretion of the Colorado beetle, *Leptinotarsa decemlineata*, contains, as major compound, a  $\gamma$ -glutamyl dipeptide of a unique non-proteinaceous amino acid which is highly toxic to ants<sup>6</sup>. In the subtribe Chrysomelina (9 species belonging to 6 genera) and in the subtribe Phyllodectina (3 species of the genus *Phratora*), the secretion contains isoxazolinone and nitropropanoic glucosides which are both toxic and deterrent to ants<sup>19</sup>.

In the subtribe Chrysolinina (13 species of *Chrysolina* and 8 species of *Oreina*), most but not all species (see below) secrete complex mixtures of cardenolides. In a single species, more than 10 different compounds can be present simultaneously<sup>22,31</sup> (Rowell-Rahier et al., in prep.). These are found in the secretion either free or linked to one, two or three monosaccharide moieties, pentoses or hexoses. Some of them, e.g. lyxose, ribose, and allose, are rarely found in secondary metabolites. So far 22 different cardenolides have been identified. They differ in their steroid moieties (6 structural types identified), or in their monosaccharides (of which 8 have been identified). Many more cardenolides may exist by permutations of the aglycone and sugar combination alone.

Within the subtribe Chrysolinina we have found to date two sets of exceptions to the general trends of secretion of cardenolides. Firstly, *Chrysolina hyperici*<sup>5</sup>, *C. varians*, *C. brunsvicensis* and *C. geminata* (Randoux et al., in prep.) secrete closely related polyoxygenated steroid glucosides. Secondly, *Oreina cacaliae* secretes pyrrolizidine alkaloids in the N-oxide form<sup>24</sup>.

All the members of the subtribe Chrysolinina secrete non-toxic ethanalamine in addition to the major compounds mentioned above. The function of ethanalamine may be to increase the water solubility of the toxins<sup>31</sup>, which reach concentrations greater than  $10^{-1}$  M<sup>22</sup>.

In the subfamily Criocerinae, preliminary information on the secretion of *Lilioceris lili* suggest that its major

component is an amino acid derivative, in which phenylalanine is linked to an unidentified compound (Randoux et al., unpubl. results).

#### Biosynthetic origin and host plant influence on defensive chemistry

The degree of diversity of defensive chemistry found in the tribe Chrysomelini is unique among comparable insect taxa. In this section, we speculate that the long parallel evolution between these specialist herbivores and their host plants may provide cues for the understanding of the evolution of such diversity.

The data summarized in the previous section show that amino acid derivatives are present as major or minor components of the defensive secretion in all the taxa examined. This is exemplified by the occurrence of the  $\gamma$ -glutamyl dipeptide in *Leptinotarsa decemlineata* and of the phenylalanine derivative in *Lilioceris lili*. For the nitropropanoic acid and isoxazolinone moieties of the Chrysomelina and *Phratora* we suggest, and are investigating, a biosynthetic pathway based on aspartic acid (fig. 4). An analogous biosynthetic pathway for nitropropanoic acid has recently been demonstrated in fungi by Baxter et al.<sup>2</sup> The ethanolamine present in the secretion of the Chrysolinina is probably derived from L-serine by decarboxylation<sup>16</sup>. It is likely that all these amino acid derivatives are synthesized from ubiquitous amino acids, and not sequestered from their host plants. The widespread occurrence of de novo synthesis of amino acid derivatives suggests that it may represent the primitive condition in the defensive chemistry of leaf beetles. The major chemicals (steroid derivatives or alkaloids)

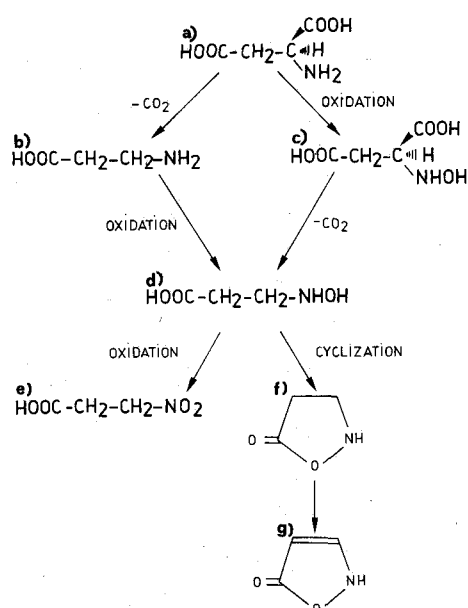


Figure 4. Hypothetical biosynthesis of isoxazolinone and nitropropanoic acid. a) L-aspartic acid; b)  $\beta$ -aminopropanoic acid; c) N-hydroxyaspartic acid; d) N-hydroxy- $\beta$ -aminopropanoic acid; e)  $\beta$ -nitropropanoic acid; f) isoxazolidin-5-one; g)  $\Delta^3$ -isoxazolin-5-one.

Table 2. Chemical defense in *Chrysolina* species feeding on *Hypericum*

Subgenus <i>Hypericia</i>	
<i>C. dydimata</i>	Sarmentogenin <sup>1</sup>
<i>C. brunsvicensis</i>	Polyoxygenated steroids <sup>2</sup>
<i>C. hyperici</i>	Polyoxygenated steroids <sup>2</sup>
<i>C. geminata</i>	Polyoxygenated steroids <sup>2</sup>
Subgenus <i>Sphaeromela</i>	
<i>C. varians</i>	Polyoxygenated steroids <sup>2</sup>

<sup>1</sup> Other components are possibly present in the secretion; <sup>2</sup> Ethanolamine also present.

found in the secretions of the Chrysolinina bear no a priori relationship to this amino acid based pattern. The influence of host plants on the nature of the secretions are probably among the major factors having prompted these departures from the primitive condition suggested above.

For example, *Oreina cacaliae* and *O. speciosissima* secrete pyrrolizidine N-oxides and are specialized herbivores on asteraceous plants known to contain precisely these compounds<sup>23, 24</sup>. The detailed relationship between *Oreina cacaliae* and *O. speciosissima* and the different alkaloids present in the Asteraceae is being investigated.

The four species of *Chrysolina* which secrete polyoxygenated steroid glucosides feed exclusively on *Hypericum*. Among those species three are closely related, but *C. varians* belongs to another subgenus, suggesting two independent colonizations of *Hypericum* by ancestors of these insects (table 2). The presence of polyoxygenated steroids in the glands thus seems to be correlated with dietary specialization on *Hypericum*. It is not known whether these steroids, which have some structural resemblance to ecdysone, are biosynthesized de novo from ubiquitous phytoesterol by the beetles, or derived from some more elaborated steroid characteristic of *Hypericum*. If the latter were the case, it would indicate that the production of polyoxygenated steroids is a secondary event in the evolution of leaf beetle defensive chemistry, resulting from host plant influence, as in *Oreina cacaliae*. This hypothesis is presently under scrutiny. Interestingly, one species (*Chrysolina dydimata*), feeding on *Hypericum* and classified in the same subgenus as *C. hyperici*, *C. brunsvicensis* and *C. geminata*, still produces the cardenolide sarmentogenin like other members of the subtribe (table 2). This suggests that *Hypericum* feeders may have evolved from cardenolide-producing ancestors.

The Chrysolinina producing cardenolides feed on diverse host plants, none of them known to contain cardenolides<sup>18</sup>. Van Oycke et al.<sup>30</sup> were able to demonstrate that in *Chrysolina coeruleans* the cardenolides are biosynthesized from cholesterol, following a pathway which is analogous to that found in plants. Moreover, the sugar moieties of the cardenolides do not correspond to those present in the beetles' host plants (Daloz et al., unpublished results).

It is not easy to understand the evolution of the biosynthesis of cardenolides in glands which synthesized amino acid derivatives in their plesiomorphic condition. At

present we can not suggest any concrete hypothesis to explain this fact. But we suggest that in addition to the identification of the major compounds found in the secretions, chemical analyses should also focus on minor compounds which could give clues to possible intermediates as well as to the full synthetic abilities of the glands. For example, lipids are found in the secretion of *Gastrophysa*<sup>14</sup> and other Chrysomelina, and in trace amounts in the Chrysolina and in *Leptinotarsa*<sup>21</sup>. The glands are thus able to produce both lipids and amino acid derivatives. It is possible that the defensive glands are derived from dermal glands which function to produce compounds involved in the maintenance of the integument. These compounds could include, for example, lipids necessary to keep the lipophilic properties of the cuticle and amino acid derivatives to act as antibiotics. Non-protein amino acids such as those found in the secretion of *Leptinotarsa* are indeed known for their antibiotic activity<sup>1</sup>.

#### Why are Chrysomelidae so often aposematic?

Defensive strategies are numerous<sup>10</sup>. Why is the combination of aposematism and toxins particularly successful in leaf beetles? We suggest that the feeding habits of these beetles were an important factor in the evolution of their mode of defense. In the Northern temperate zones, Chrysomelidae are mostly specialized on herbaceous plants or early successional shrubs (e.g. Salicaceae and Betulaceae). These are the kind of plants which are also often protected by toxins acting at low concentrations<sup>11,26</sup>. Indeed, Brown<sup>3</sup> even suggested that aposematic insects are good indicators of medicinal plants, on the rationale that many aposematic insects sequester their toxins from their host plants<sup>27</sup>. These plant toxins often cause delayed food poisoning in vertebrate predators; this poisoning is associated with strong negative conditioning<sup>13</sup>. Bright colors of the prey will reinforce this associative learning.

As discussed above, the influence of host plants on the defensive chemistry of the leaf beetles seems to be a secondary event in their evolution. The ancestral insects were probably already aposematic while still producing their toxins by de novo biosynthesis. Thus, in this group the sequestration of plant toxins was not a prime factor in the evolution of aposematism.

We suggest that the feeding habits of the beetles make them apparent to predators such as birds, and that visual aposematism linked with the production of toxins offers the best protection against such predators (see Van Oycke<sup>30</sup> for similar discussion). Chrysomelinae have a low mobility once they have found a host plant and also a high reproductive rate. Thus, these oligophagous beetles tend to form large aggregations on patchy food plants in open habitats. Birds are known to search for prey more intensively in the area of their last rewards<sup>4</sup> and to develop search images quickly<sup>29</sup>. A rapid and powerful negative conditioning of the birds by the aggre-

gated beetles is thus of critical importance to their survival. In this case, we should expect to find more aposematic insects among oligophagous herbivores feeding on herbaceous plants in open habitats than among polyphages feeding on forest trees. This seems to be true not only for Chrysomelidae but also for other phytophagous insects<sup>20</sup>. The unsealed way of life of Chrysomelidae allows them to feed at the top of plants on young nutritious leaves. This exposed position is not without danger such as overheating or damage due to increased UV light. Colors, and especially metallic colors, possibly provide some protection against the deleterious effect of direct sunshine. There is an avenue open to research on this possible role of colors in aposematic insects.

#### Conclusion

Although our knowledge of the evolution of chemical defense in the Chrysomelidae is still very restricted, some general patterns are emerging, which suggest that the phytophagous habits of the beetles have strongly influenced their mode of defense. First, specialization on herbaceous plants in open habitats favored the joint evolution of defense by aposematic coloration and by toxins. Secondly, shifts in host plant affinities and subsequent adaptation of the beetles to the plant toxins were responsible for spectacular changes in the defensive chemistry of otherwise morphologically stable glands.

We admit willingly that the ideas put forward in this review are mostly speculative and based on little evidence. We hope that they will stimulate further research and the discussion of alternative interpretations of the data.

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## Insect chemical ecology. Summary and concluding remarks

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The papers presented in this Multi-author Review provide an eclectic, rather than a comprehensive, overview of research progress within the diverse and dynamic field of insect chemical ecology. Each paper, within its subject area as defined by the author, serves as a bridge between the current state of knowledge and the next conceptual step which will be made possible by data gathered from ongoing investigations as this Review is published. In spite of the tremendous differences in subject matter between the papers, certain linkages arise from a careful examination of the collection.

Chapman and Bernays point out that direct observations of insects on plants tend to be uncommon, but are important if we are to understand the processes by which insects accept host plants. They rightly emphasize the leaf surface as the interface between the insect's battery of chemoreceptors (both olfactory and gustatory) and the plant, and suggest that the decision to accept or reject frequently occurs even before an insect takes the first bite. Renwick reaches a similar conclusion with respect to the importance of leaf surface chemistry in oviposition

by insects. Both papers raise the point that acceptance/rejection is the result of a concatenation of behaviors, which can rarely be ascribed to a single, unique plant chemical. In the case of oviposition, volatile substances, in some cases acting in concert with visual stimuli, attract the insect from a distance. It is only once the insect has alighted on the plant that the surface chemicals ('arrestants') can come into play in determining the insect's ultimate decision of whether to accept or not.

The Diabroticite beetles provide a strong case in point. Metcalf and Lampman explore the evolutionary ties between this taxa and their host plants, the Cucurbitaceae. It is the unique triterpenoids, the cucurbitacins, which characterize this family of plants in the chemical sense, and these allelochemicals are powerful arrestants and feeding cues for the beetles. However, it is a blend of somewhat nonspecific floral volatiles which serve to attract the beetles to the plants. This beetle-allelochemical interaction includes two further points of interest: (1) because the cucurbitacins are such potent and specific arrestants for these insects, this knowledge has been of