

TOXINS IN CHRYSOMELID BEETLES

Possible Evolutionary Sequence from De Novo Synthesis to Derivation from Food-Plant Chemicals

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Abstract—In the Chrysomelinae, it appears that de novo synthesis of chemicals for defense is the primitive state, and the sequestration of plant chemicals for defense the derived state. The derived state evolved through both the morphological and biochemical preadaptiveness of the homologous defensive glands. In the adults, we discuss one unique case of sequestration in exocrine defensive glands of host-plant pyrrolizidine alkaloids by *Oreina cacaliae*. However, hypericin is not sequestered either in the glands or elsewhere in the body of *Chrysolina* spp. feeding on *Hypericum*, which contradicts an earlier claim. In the larvae, we examine in more detail how the phenolglucoside salicin can be used as the precursor of the salicylaldehyde present in the defensive secretion of *Phratora vitellinae* and *Chrysomela* spp. with minimal changes in the biochemical mechanisms involved in the biosynthesis of iridoid monoterpenes in related species.

Key Words—Coleoptera, Chrysomelidae, beetle, sequestration from plants, defense, pyrrolizidine alkaloids, salicin, salicylaldehyde, iridoid monoterpenes, hypericin, *Oreina cacaliae*, *Phratora vitellinae*, *Chrysomela* spp.

INTRODUCTION

Leaf-beetles provide us with striking examples of brilliant coloration. In many chrysomelid species, this conspicuousness is clearly fortified by unpalatability derived from chemical defense in both larval and adult stages. Upon alarm, these defensive chemicals can be released from specialized exocrine glands, by reflex bleedings, or by enteric discharges. In some cases, it is the mechanical action of the predator itself that causes the defensive substance to ooze from wounds of the prey.

The morphology, biology, and chemistry of such defenses in the Chrysomelidae has recently been reviewed (Pasteels et al., 1988) and will not be discussed in further detail here. The major point of interest in this paper is the derivation of chemical defense from host plants from a biochemical as well as evolutionary point of view. The Chrysomelidae are a relatively well-studied group that are known to biosynthesize their own chemical defenses (e.g., iridoids, cardenolides, isoxazolinones) (Pasteels et al., 1988) or to sequester their defenses from a food plant (e.g., salicylaldehyde, juglone, cucurbitacins) (Ferguson and Metcalf, 1985; Pasteels et al., 1989a). This base of comparative knowledge provides a platform from which we can begin to assess the processes involved in the evolution of the diversity of chemical defenses found in the taxon.

Here we review our recent work on plant-derived toxins in the defensive secretion of the Chrysomelinae. More particularly, we postulate the biochemical mechanisms that have allowed the replacement of autogenous compounds by plant-derived toxins by some taxa in the course of evolution.

Some general information on the nature and occurrence of the defensive glands is required before a more detailed discussion of the derivation of defense can be given. Defensive exocrine glands are found in both larval and adult leaf beetles. However, these two sets of glands are not homologous, and their distribution is restricted to certain taxa (Deroe and Pasteels, 1982; Pasteels et al., 1988). Moreover, when they occur in both larval and adult stages of the same species, they secrete toxins of completely different chemical natures. So far, no species is known in which plant-derived toxins occur in the defensive glands of both larvae and adult defensive glands. Yet, in both adults and larvae of genetically closely related species, the same morphological structures are utilized either to synthesize autogenous toxins or to store plant-derived toxins. This genetic relatedness, in conjunction with differences in the identity of chemical defenses, particularly in homologous glands, provides an opportunity to determine the influence of the host plant on defensive chemistry.

In the first part of this paper we report on a previously undescribed and striking example of host-plant influence on the exocrine secretions of adult Chrysomelinae (*Oreina* and pyrrolizidine alkaloids) and reconsider briefly a

previous report of sequestration (*Chrysolina* and hypericin). In the second part, we examine how plant toxins are sequestered in larval secretion (e.g., *Phratora* and *Chrysomela*, and phenolglucosides) and relate this to the probable mode of evolution of this habit.

HOST-PLANT INFLUENCE ON ADULT DEFENSE

In adults of the subfamily Chrysomelinae, the defensive glands are situated in the pronotum and the elytra, especially along the lateral margins. After physical disturbance, the secretion oozes from the gland pores and collects in marginal grooves as well as pronotal and elytral depressions, which help to retain the secretion on the insect. Each gland is formed from a group of secretory cells opening into a common duct. The secretion accumulates within the gland cells themselves, as there is no glandular reservoir. Within this taxon, these glands are morphologically very similar and undoubtedly homologous (Deroe and Pasteels, 1977, 1982). Comparative chemical studies have demonstrated a remarkably diversified defensive chemistry, including cardenolides, dipeptides, and isoxazolinone glucosides. The types of defensive compounds found in the different subtribes studied are summarized in Table 1. There is a close parallel between current classification and the type of compound secreted (see Pasteels et al., 1988).

In this section we will consider only the adults of the subtribe Chrysolina, which, with few known exceptions to date, produce cardenolides and ethanolamine. In most species of the two European genera of this subtribe, the secretion contains a diversity of cardenolides (Table 2). The structure of 22 different cardenolides has been elucidated (Van Oycke et al., 1988). These cardenolides differ either in their aglycones, of which six are structurally identified,

TABLE 1. DISTRIBUTION OF ADULT DEFENSIVE COMPOUNDS MOST FREQUENTLY FOUND IN SUBTRIBES OF CHRYSOMELINAE (Chrysomelidae, Coleoptera)

Chrysomelinae subtribes	Defensive compounds
Chrysolina 2 genera, 21 spp.	Cardenolides and ethanolamine
Doryphorina 1 sp.	γ -Glutamyl dipeptide
Chrysomelina 6 genera, 9 spp.	Isoxazolinone and nitropropionic acid glucosides
Phyllodectina 1 genus, 3 spp.	

TABLE 2. DEFENSIVE CHEMISTRY OF ADULT CHRYSOLININA PRODUCING CARDENOLIDES

Beetles	Host plants	Secretions
<i>Oreina</i> 8 spp.	Apiaceae Asteraceae	22 cardenolides identified 6 aglycones 8 sugars or esterified derivatives
<i>Chrysolina</i> 13 spp.	Lamiaceae Ranunculaceae Plantaginaceae Scrophulariaceae Hypericaceae	up to 138 $\mu\text{g}/\text{insect}$ (2×10^{-1} M to 4×10^{-1} M) also present: ethanolamine

or in their sugar moieties, of which eight have been elucidated. Cardenolides are found in at least eight species of *Oreina* and 13 species of *Chrysolina* (Pas-teels et al., 1988). This widespread occurrence suggests that this is the plesiom-orphic condition in the taxon. The host plants of the beetles belonging to these two genera are quite diverse, including seven different families of plants (Table 3). None of these host plants contains cardenolides. Van Oycke et al. (1987) were able to demonstrate that in *C. coeruleans* the cardenolides are biosynthe-sized from cholesterol, following a metabolic pathway that is identical to that found in plants. The function of ethanolamine may be to increase the solubility of the cardenolides in the secretion to reach concentration greater than 10^{-1} M.

TABLE 3. HOST-PLANT RELATIONSHIPS AND CHEMICAL DEFENSES OF ADULT CHRYSOLININA

Beetles	Host plants	Secretions ^a
12 spp. of <i>Chrysolina</i>	<i>Mentha</i> , <i>Lycopus</i> , <i>Rosmarinus</i> , <i>Galeopsis</i> , <i>Glechoma</i> , <i>Ranunculus</i> , <i>Plantago</i> , <i>Linaria</i>	Cardenolides
<i>Chrysolina dydimata</i>	<i>Hypericum</i>	Cardenolides
3 other spp. of <i>Chrysolina</i>	<i>Hypericum</i>	6-Oxosteroids
7 spp. of <i>Oreina</i>	<i>Chaerophyllum</i> , <i>Peucedanum</i> , <i>Centaurea</i>	Cardenolides
<i>Oreina speciosissima</i>	<i>Senecio</i> , <i>Adenostyles</i> , <i>Petasites</i>	Cardenolides
<i>Oreina cacaliae</i>	<i>Senecio</i> , <i>Adenostyles</i> , <i>Petasites</i>	Pyrrolizidine alkaloids

^a Ethanolamine is also present in all secretions independently of the type of compound secreted.

There are, however, two striking exceptions to this pattern: three species of *Chrysolina* feeding on *Hypericum* secrete 6-oxosteroids and one species of *Oreina* feeding on various Asteraceae secretes pyrrolizidine alkaloids, in addition to ethanolamine. None of these insects secrete cardenolides.

In *Oreina cacaliae*, the major component of the secretion is seneciphylline *N*-oxide (Pasteels et al., 1989b). This species feeds on three genera of Asteraceae, all known to contain the pyrrolizidine alkaloid seneciphylline in its *N*-oxide form. Although sequestration has not yet been demonstrated unambiguously, the presence of exactly the same compound in the plant and in the beetle is unlikely to be a mere coincidence. Thus, secretion of autogenous cardenolides appears to have been replaced by sequestration of plant toxins; but ethanolamine is still produced by the beetle. More mystifying is *O. speciosissima*, a closely related sympatric species, which feeds on the same set of plants but secretes cardenolides rather than pyrrolizidine alkaloids. There are at least three possible alternative and not exclusive hypotheses to account for this. First, it may be advantageous to keep the secretions as chemically diverse as possible to avoid adaptation of specialized predators. Second, the secretion of cardenolides may be the more primitive condition, and thus, *O. speciosissima* has not yet developed the ability to sequester plant toxins. Thirdly, the defensive secretions may also have some species-specific pheromonal function.

Three of four species of *Chrysolina* feeding on *Hypericum* form the second exception to the rule of secretion of autogenous cardenolides (see Table 3). *Chrysolina* feeding on *Hypericum* produce as their main chemical defense polyoxygenated steroids characterized by a ketonic function on carbon 6 as in ecdysone (Daloze et al., 1985; Randoux et al., in preparation). The metabolic origin of these compounds is still unknown, although it is reasonable to hypothesize that they are metabolites of sequestered plant sterols.

There is a persistent claim in the literature that *C. brunsvicensis* sequesters the plant quinone hypericin. This claim is based on a short report by Rees (1969), which states that the quantity of hypericin found in the beetle was too high to be contained solely in the gut. Hypericin, however, is present neither in the defensive secretions containing polyoxygenated steroids, nor is it present in the hemolymph or other body parts of *C. brunsvicensis*, *C. varians*, *C. hyperici*, and *C. geminata* in amounts that could significantly contribute to defense ($<0.5 \mu\text{g}/\text{beetle}$) (Duffey and Pasteels, in preparation).

Thus, it seems that certain beetle species have evolved to replace their primitive de novo secretion with sequestered plant toxins. The reasons for this replacement are not yet known and await a better understanding of both the ecological and metabolic factors involved. This information is not yet available for adult beetles but, as we will see in the next section, it is starting to emerge for the larvae.

HOST-PLANT INFLUENCE ON LARVAL DEFENSE

The larvae of the subtribe Chrysomelina and of the genus *Phratora* possess nine pairs of segmental exertile glands (Pasteels et al., 1984). Many species produce highly reactive iridoid monoterpene aldehydes and ketones (Table 4). These species feed on a great diversity of host plants (seven families). The plants are not known to contain iridoid precursors, and the iridoid monoterpenes must be synthesized de novo by the larvae. Following the same reasoning as for adult defenses, we suggest that this widespread occurrence of autogenous iridoid monoterpenes represents the primitive condition. Some species, however, produce aromatic compounds such as salicylaldehyde, juglone, and phenylethyl esters, instead of iridoid monoterpenes. The metabolic origin of the phenylethyl esters produced by the North American *Chrysomela interrupta* remains to be clarified (Blum et al., 1972). The other shifts in defensive chemistry can be correlated with host-plant influence. For example, species producing salicylaldehyde feed on *Salix* and *Populus*, which are rich in salicin and from which salicylaldehyde is derived (Rowell-Rahier and Pasteels, 1982; Pasteels et al., 1983; review in Rowell-Rahier and Pasteels, 1986; Pasteels et al., 1989a). Note that in the genus *Phratora*, some species produce autogenous iridoid monoterpenes, but *Phratora vitellinae* derives salicylaldehyde from host-plant salicin, although all species feed on Salicaceae. Thus, as observed in the adults (see above), species belonging to the same genus and feeding on the same host plants may either derive their toxins from host plants or biosynthesize them de novo. The hypotheses advanced to explain this double origin of toxins in the

TABLE 4. DISTRIBUTION OF DEFENSE COMPOUNDS IN CHRYSOMELINAE LARVAE

Beetles	Host plants	Secretions
Subtribe Chrysomelina 6 genera, 11 spp.	Salicaceae Betulaceae Ranunculaceae Apiaceae Polygonaceae Scrophulariaceae Cruciferae	Iridoid monoterpenes
<i>Chrysomela interrupta</i> 6 spp. of <i>Chrysomela</i>	Betulaceae Salicaceae	Phenylethyl esters Salicylaldehyde $\pm$ benzaldehyde
<i>Gastrolina depressa</i>	Juglandaceae	Juglone
Subtribe Phyllodectina 3 spp. of <i>Phratora</i> <i>Phratora vitellinae</i>	Salicaceae Salicaceae	Iridoid monoterpenes Salicylaldehyde

adults are also valid for the larvae. Finally, *Gastrolina depressa* probably derives its juglone (Matsuda and Sugawara, 1980) from the host plant *Juglans*.

To derive salicylaldehyde from salicin, only two enzymes are needed; first, a β -glucosidase to hydrolyze the β -glucoside salicin to the aglycone saligenin and glucose; second, an oxidase to transform saligenin into salicylaldehyde. To derive juglone from the plant toxin, exactly the same two kinds of enzymes are necessary. Likewise, we hypothesized that in the species that secrete iridoid monoterpenes, these chemicals are, initially, produced as glucosides; these are less toxic, more stable, and easier to transfer across membranes (from gland cells to lumen of exertile gland). In the second step, after secretion of the glucosides into the lumen, it is hypothesized that the glycoside is enzymatically hydrolyzed to the hemiacetal and then subsequently oxidized to the dial (e.g., plagodial) (Figure 1). In all these above cases, the common factor is the concurrent action of a β -glucosidase and an oxidase upon a glucoside and an alcohol, respectively, to liberate a carbonyl-containing defensive chemical. Hence, if one views the de novo synthesis of defense chemicals as the primitive state, the enzymes are already (i.e., preadaptively) present that would permit the evolution of the use of plant-derived glucosides for defense. Such an evolution only requires changes in the specificity of the two enzymes, particularly that of the oxidase.

This evolutionary scheme has been tested indirectly (Duffey and Pasteels, in preparation) by comparing the enzymatic specificity of the β -glucosidase and oxidase in the gland fluid of *Plagiodera versicolora*, a species that feeds on

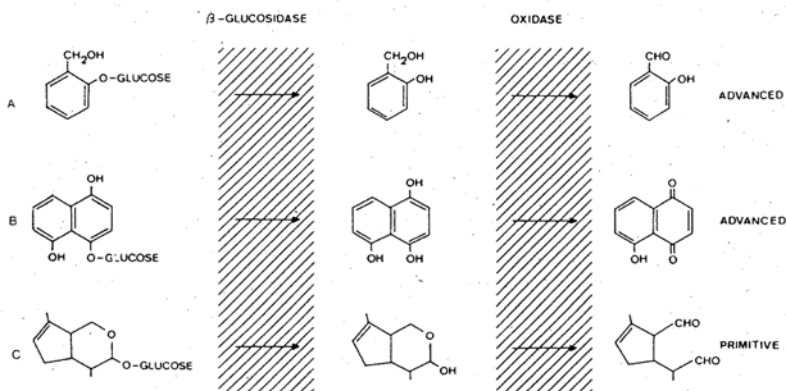


FIG. 1. Final steps in the production of toxins in chrysomelid larvae; A and B exemplify the derivation of toxins from plant precursor; C, de novo biosynthesis. B and C remain putative.

Salix and secretes autogenously monoterpenes, and of *Chrysomela populi*, a species that also feeds on *Salix* and by sequestration secretes salicylaldehyde.

Let us consider *Plagiodera versicolora* first; a hypothetical schematic representation of the glands and associated organs and processes involved in the production of iridoidal defenses of larvae is given in Figure 2. The gland cells supposedly biosynthesize and secrete into the lumen of the gland the β -D-glucoside of monoterpene iridoid. Up to now we have not been able to detect the glycosidic or aglyconic hemiacetal form of the monoterpene in the defensive secretion. However, glucose is readily detectable, which supports the hypothesis that a glucoside is a precursor. Moreover, significantly high levels of non-specific β -glucosidase activity have been detected in this fluid. This activity is able to hydrolyze a wide variety of phenolic and terpenoid glucosides (including salicin). We have not been able to prove the existence of the oxidase, possibly because of its specificity (see below for salicin) and of a lack of appropriate substrates. The secretion was not able to oxidize saligenin to salicylaldehyde or to oxidize the aglycone of the iridoid glucoside mussaenoside.

Another aspect of the scheme deserves attention. The lumen of the gland is filled with a biphasic fluid comprising an aqueous component containing the enzymes and glucose and a nonmiscible phase containing the monoterpene aldehydes. The aqueous phase permits the solution of the postulated glucoside as well as of the aglycone. We assume that once the glucoside is secreted into the

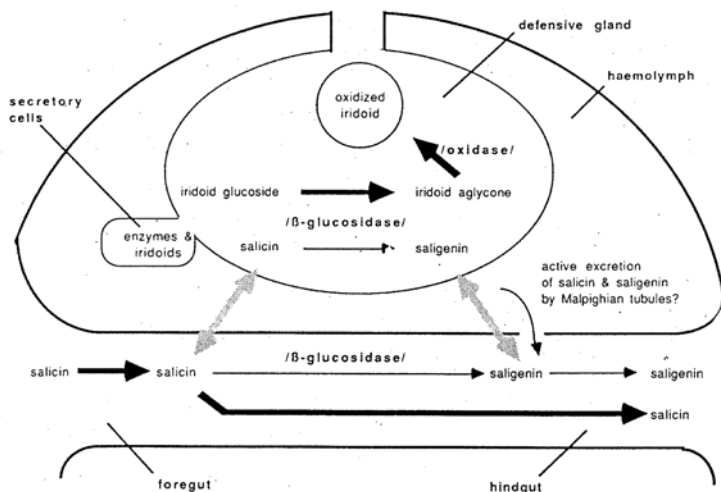


FIG. 2. Schematic representation of the biosynthesis of defensive iridoid monoterpenes in the larvae of *Plagiodera versicolora*. (Adapted from Duffey and Pasteels, in preparation.)

lumen, it is rapidly hydrolyzed, and the resulting aglycone rapidly oxidized to the dial, which is the reason the former two are not detectable. However, once the dial is formed, it rapidly forms the second phase (an oil) because of its immiscibility with the aqueous phase.

Based on the evidence from *Plagioderma versicolora*, we suspect that the major biochemical determinant of the chemical nature of the defensive secretion is the oxidase, rather than the glucosidase. In other words, a change in the specificity of the oxidase, say the acquisition of the ability to oxidize saligenin, would permit an insect to develop a switch from de novo biosynthesis of iridoid monoterpenes to sequestered defense via the utilization of the plant glucoside salicin. In *Chrysomela populi*, we believe we have evidence for this switch in defensive strategy.

In species such as *C. populi*, which produces salicylaldehyde (Figure 3), the defensive secretion is biphasic and readily emulsifiable. The aqueous phase contains not only high levels of both nonspecific β -glucosidase activity (it will hydrolyze many phenolic terpenoid glucosides) and specific oxidase activity (O_2 -dependent, NAD-independent activity against a limited number of analogs and homologs of saligenin), but also contains traces of salicin and saligenin (Duffey and Pasteels, in preparation). The determining enzyme, the oxidase, which shows high specificity, rapidly converts saligenin to salicylaldehyde but is unable to oxidize the aglycone of mussaenoside to the aldehydic form; the

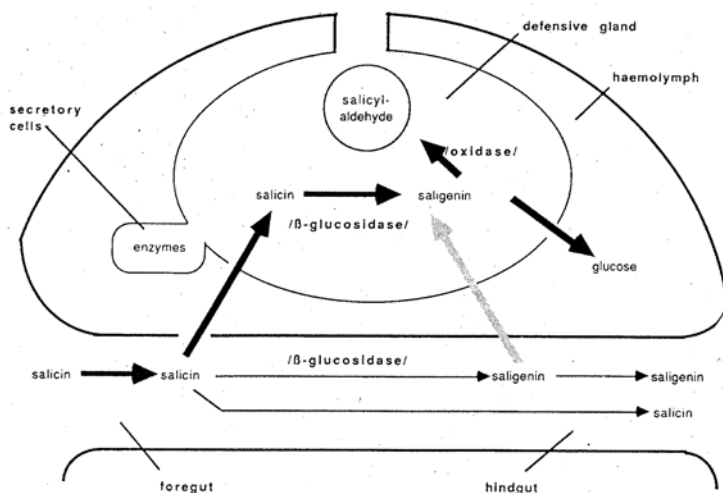


FIG. 3. Schematic representation of the biosynthesis of defensive salicylaldehyde derived from host-plant salicin in the larvae of *Chrysomela populi*. (Adapted from Duffey and Pasteels, in preparation.)

glucosidase, by contrast, effectively hydrolyzes mussaenoside. Once the salicylaldehyde is produced in the gland fluid, it forms a distinct organic phase because of its lipophilic nature and its immiscibility with the aqueous phase. The formation of a water-insoluble phase within the confines of the gland restricts the diffusion of toxic salicylaldehyde to the body in general.

There is another salient aspect of the scheme (Figure 2) that relates to how a change in the specificity of the oxidase could lead to a switch from de novo synthesis of monoterpenes to production of salicylaldehyde from a sequestered precursor. Note again that both *P. versicolora* and *C. populi* feed upon *Salix* and both ingest salicin. Some β -glucosidase activity is present in the gut of *C. populi* and *C. tremulae* (Duffey and Pasteels, in preparation; Pasteels et al., 1983); the ingested foliage does not contribute meaningfully to the production of saligenin. However, only the glands of *C. populi* produce salicylaldehyde. Both salicin and saligenin probably diffuse passively through the bodies of the insects into the gland fluid because of their water solubility. With a change in enzyme specificity towards metabolism of saligenin, resulting from gland glucosidase activity, salicylaldehyde can be accumulated rapidly because of the constant inward diffusion of plant substrates. The conversion of saligenin to salicylaldehyde may also be of benefit, considering that saligenin is toxic and must probably otherwise be eliminated by reglucosylation, excretion by Malpighian tubules, or other mechanisms. It is worth noting that the feces of *C. populi* contain only traces of salicin and saligenin, whereas the feces of adults of *C. populi* and larvae of *P. versicolora*, neither of which sequester salicylaldehyde, have very high levels of salicin and saligenin.

Thus we propose that a single change in the specificity of the oxidase is very likely the only prerequisite to the utilization of plant precursors by the larvae. The process could be further refined by an increased permeability of the gut to salicin and/or saligenin and the reduction of its active excretion by the Malpighian tubules. Additionally, more glucose will be recovered by the larvae as the quantity of salicin hydrolyzed increases, thus giving the species utilizing salicin a net energetic advantage compared to those synthesizing iridoid monoterpenes (Rowell-Rahier and Pasteels, 1986). According to our hypothesis, sequestration of plant-derived toxins is a secondary event that has replaced the more costly autogenous synthesis of toxins.

It is reasonable to suppose that those larvae that secrete juglone underwent an analogous switch in the specificity of the oxidase.

CONCLUSIONS

Our results suggest that during the evolution of leaf-beetles, the colonization of new host plants by these specialized herbivores had major consequences on their chemical defenses. On at least four independent occasions

(once in the adults and three times in the larvae), the sequestration of plant toxins or the derivation of toxins from host-plant precursors has replaced the production of autogenous compounds, although the defensive glands, used both as storage and releasing organs, remained basically unchanged.

Our present understanding of the metabolic processes involved during the derivation of toxins from plant precursors in larval secretions not only illuminates their probable evolution, but also points to the likely limitations of plant influence on larval chemical defense.

Evolutionary success was probably the result of lower defensive costs. Preexisting glands and enzymes (β -glucosidase and oxidase), only slightly modified in their specificity, are used to derive toxins from plants, and thus no additional cost is required for the metabolism of plant precursor by the larvae, and, additionally, there is an economy in toxin biosynthesis and, possibly, in active excretion of the plant toxin. Moreover, the plant toxin acquires a nutritive value due to the glucose released during its metabolism. This is possible only if the plant precursor is a glucoside, the aglycone of which can be transformed by oxidation into a nonpolar toxin. Host-plant influence on larval secretion may be restricted to glucosides. So far, only plant glucosides are known to be used for defense by the larvae, and twice (in *Chrysomela* and *Phratora*) it is the same glucoside, salicin, that is used.

The adult glands must function on completely different principles, which remain poorly understood; plant glucosides are not metabolized in those glands, even in the species that use these glucosides for defense in their larval stages. At present it is impossible even to guess why the sequestration of pyrrolizidine alkaloids is possible in glands that originally biosynthesized cardenolides from cholesterol or why sequestration of pyrrolizidine alkaloids has evolved repeatedly in insects (review in Boppré, 1986).

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