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Chemical defence in the larvae of the leaf beetle *Gonioctena viminalis* L. (Coleoptera: Chrysomelidae)

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Summary. The leaf beetle larva of *Gonioctena (Phytodecta) viminalis* L. has been shown to produce five volatile constituents within its paired abdominal defensive gland reservoirs. It is the first time that these compounds have been reported to occur in coleopteran defensive glands (linalool, phenylethanol) and Chrysomelidae larvae (6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, 2-hexenal). In addition to the gross morphology of the *Gonioctena* gland and its discharge behavior, the natural products found are discussed in terms of chemotaxonomy.
Key words. Defensive secretions; glands; Chrysomelidae; Coleoptera; phenylethanol; linalool; 6-methyl-5-hepten-2-one; 6-methyl-5-hepten-2-ol; 2-hexenal; chemotaxonomy.

In phytophageous Chrysomelidae or leaf beetles, each developmental stage, namely eggs[1], larvae[2], pupae[3] and adults[2], may be chemically protected. Up till now the paired eversible defensive glands which are located on meso- and metathorax and abdominal segments have been the subject of various investigations both with respect to gland morphology and the chemistry of the secretions[2]. But a considerable number of leaf beetle larvae which belong to Paropsina, Gonioctenina, Doryphorina and Chrysolinina are characterized by having only one pair of abdominal defensive glands which substitute for the serial defensive glands. These single paired defensive glands have not been studied morphologically and chemical data are only available from the larvae of the Australian, eucalyptus-defoliating species *Paropsis atomaria* Ol.; they secrete hydrogen cyanide, benzaldehyde, glucose[4] and mandelonitrile which are derived from prunasin[5] which is probably biosynthesized by the beetle, because it does not occur in the larval food plant. We now present, for the first time, a European species, the *Salix*-feeding *Gonioctena (Phytodecta) viminalis*, which possesses single paired larval defensive glands. We describe the gland structure and the chemical composition of the *Gonioctena viminalis* larval secretion, and want to place special emphasis on the chemotaxonomic data.

Materials and methods. Gregarious feeding larvae of *Gonioctena (Phytodecta) viminalis* L. were caught on the leaf surface of *Salix* trees in the bog area 'Hohes Venn' (Belgium). The identity of the species was confirmed by breeding. While being molested by carefully touching with forceps the *Gonioctena* larva was induced to push its abdominal tip on to a minute moist triangle of filter paper. The cooled filter paper triangle with the adhering defensive secretion was inserted into the groove of a movable wire plunger of a 0.1 µl mini-injector (Precision Sampling Corporation) and injected into a gas chromatograph[6]. This method allowed splitless injection of the larval secretion without using any solvent. The secretions from five to ten third stage *Gonioctena viminalis* larvae had to be collected in order to obtain one gas chromatogram. The secretions were analyzed by both gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) (GC: Carlo Erba Fractovap 2900; GC-MS: Varian 3700 coupled to a Varian MAT 44 S quadrupole mass spectrometer, 80 eV; 8 m CW 20 M glass capillary column, temperature program: 65–165 °C: 5 °C/min; 165–220 °C: 10 °C/min).

Results. When feeding on *Salix* leaves the three larval stages of *Gonioctena viminalis* exhibit an interesting form of defensive behavior. Following vigorous molestation (fig. 1A), the black larva suddenly bends its hindbody dorsally. Between the seventh and eighth abdominal tergit, two red-colored vesicles are everted for a short time; these bear droplets of a refreshingly smelling defensive secretion. Simultaneously a soft, bright red-colored membrane surrounding the anal region is everted.

The abdominal vesicles show a roughly sculptured surface compared with the relatively smooth intersegmental membrane (fig. 1B, C) which is characteristic for membranes where volatile compounds evaporate[7]. The everted, boot-shaped vesicles (fig. 1B) represent glandular reservoirs, apically supplied with numerous ovoid glandular units per vesicle. Like the larval segmental gland cells of Chrysomelina and Phyllodectina, the voluminous gland cells of *Gonioctena* are part of the reservoir epithelium. Preliminary results reveal the presence of a curved chitinous channel through which the secretion reaches the reservoir. The retraction of both vesicles after molestation is effected by at least four muscles whose exact structure has not yet been studied in detail.

The intensive-smelling droplet of the defensive secretion of *Gonioctena viminalis* contains a major component **2** with a molecular mass of 126 (fig. 2). Both the EI-mass spectral pattern of constituent **2** (fragments at m/z 126 M^+, 111, 108, 93, 83, 71, 69, 58, 55, 43, 41) and the comparison of retention values with an authentic sample of 6-methyl-5-hepten-2-one indicate that **2** represents 6-methyl-5-hepten-2-one. This ketone, which comprises 90–98% of the total peak area per larva, is accompanied by traces of the biogenetically related 6-methyl-5-hepten-2-ol (com-

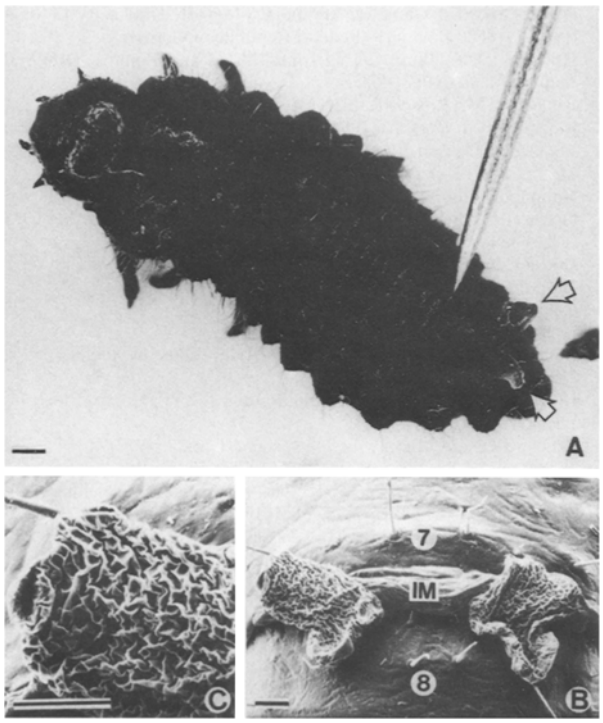


Figure 1. *A* Third stage larva of *Gonioctena viminalis* L. everting abdominal vesicles (arrows) on molestation. *B* Surface view (SEM-photograph) of abdominal tergites 7 and 8 with everted vesicles and a smooth intersegmental membrane (IM). *C* Enlargement of an everted vesicle, surface view (SEM-photograph). Bars A: 0.5 mm; B, C: 100 µm.

pound **3**; EI-fragments at m/z 128 M^+, 110, 95, 85, 81, 71, 69, 55, 43, 41). The remaining three trace constituents **1**, **4** and **5** from the *Gonioctena viminalis* secretion were identified by comparing their EI-mass spectral data with the recorded mass spectra of authentic compounds. Constituent **1** showed a molecular mass of 98 and its EI-mass spectrum (fragments at m/z 98 M^+, 97, 83, 80, 70, 69, 57, 55, 42, 41) was identical to authentic trans-2-hexenal. The mass spectral data of compound **4** and authentic linalool (M^+: 154) were indistinguishable (fragments of **4** at m/z 154, 136, 121, 107, 93, 80, 71, 69, 55, 43, 41). The mass spectrum of compound **5** exhibited a base peak at m/z 91 (tropylium ion) and was identical to the mass spectrum of authentic phenylethanol (fragments of **5** at m/z 122 M^+, 103, 92, 91, 77, 65, 63, 51).

Discussion. Other than in the primarily glandless Timarchini, larval defensive glands are present in representatives of Chrysomelini (fig. 3). Here it is tentatively assumed that the glands of all Chrysomelini larvae may principally be homologously indepen-

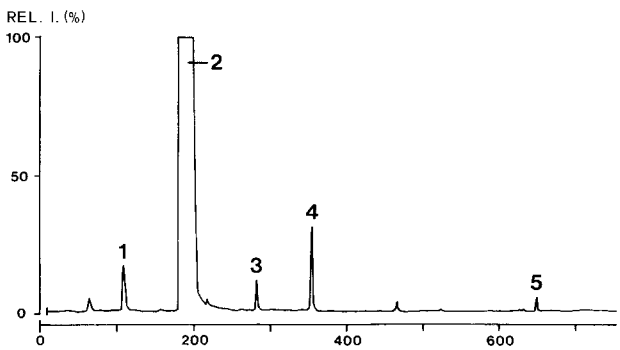


Figure 2. Part of the total ion current chromatogram of the defensive secretion of the leaf beetle larva *Gonioctena viminalis* (x-axis: number of recorded mass spectra; **1**: 2-hexenal, **2**: 6-methyl-5-hepten-2-one, **3**: 6-methyl-5-hepten-2-ol, **4**: linalool, **5**: phenylethanol).

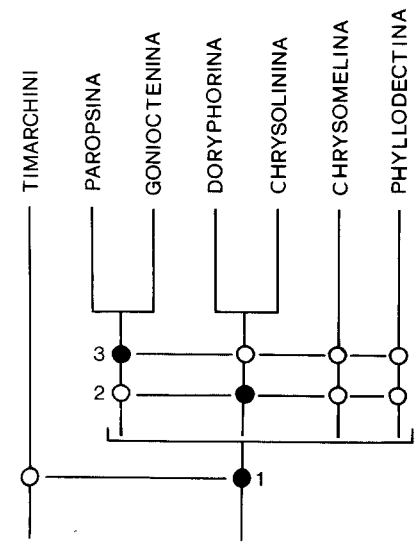
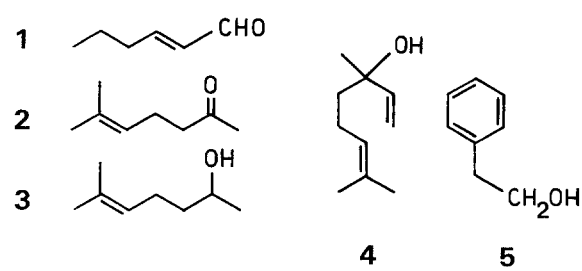


Figure 3. Cladogram of Chrysomelinae (= Timarchini + Chrysomelini) based on larval defensive glands (taxa according to Daccordi[2]; black circles indicate the derived, open circles the primitive (here absent) character state; see text).

dent of their morphological occurrence, since they possess the same peculiar fundamental structure consisting of only a few, extremely voluminous gland cells which are located beneath a distinct reservoir area[7], as found histologically in no other coleopteran family[8] (derived character 1 in fig. 3). It may be that the serial, dorsolaterally-located gland reservoirs of Chrysomelina and Phyllodectina represent a primitive condition which has been reduced to give rise to the one pair of vesicles situated at the abdominal apex. Independently a pair of vesicles might have evolved either between tergits 7/8 (Paropsina, Gonioctenina; derived character 3 in fig. 3) or between tergits 8/9 (Doryphorina, Chrysolinina; derived character 2 in fig.3). A similar rudiment of originally segmental vesicles has been observed in Malachiidae[10]. The two types of single gland pairs appear not to be homologous. The 'Chrysolina'-group of COX[9] (which includes Gonioctenina, Doryphorina, Chrysolinina) certainly represents no natural unit.

The defensive constituents **1–5** of *G. viminalis* larvae are unique in chemically-defended leaf beetle larvae, and both linalool (**4**) and phenylethanol (**5**) have been recorded for the first time in the defensive glands of Coleoptera[11]. Linalool has been reported to be present in heteropteran scent glands[12], whereas both phenylethanol and 6-methyl-5-hepten-2-ol have been found in the exocrine glands of ants (*Camponotus* and *Iridomyrmex*[11]). In Coleoptera trans-2-hexenal, a typical heteropteran allomone, has been isolated only from the defensive glands of certain rove beetles[13] and darkling beetles[14]. 6-Methyl-5-hepten-2-one is especially known in the exocrine glands of various Hymenoptera species and has been found in the anal glands of the rove beetle *Stenus comma*[15]. In addition the presence of both compound **2** and **3** has been recorded from the defensive secretion of the rove beetle *Xantholinus glaber*[16].



The Gonioctenina secretion completely differs from the hydrogen cyanide and benzaldehyde secretion of its sister group Paropsina[4]; these compounds are derived from mandelonitrile and the cyanogenic glucoside prunasin[5] which is probably biosynthesized by the beetle. But the *Gonioctena* larvae on the other hand produce three defensive compounds (**2**, **3**, **5**) which show biogenetic affinities for typical allomones of Chrysomelina and Phyllodectina. Phenylethanol (**5**) is a precursor for 2-phenylethyl isobutyrate and 2-phenylethyl 2-methyl butyrate in the defensive secretion of *Chrysomela interrupta*[17]. 6-Methyl-5-hepten-2-one (**2**) and the corresponding alcohol (**3**) may be biogenetically derived from citral, a major precursor of cyclopentanoid monoterpenes[11] which are present abundantly in the defensive secretions of both Chrysomelina and Phylodectina[2]. The multifarious occurrence of *Gonioctena* compounds supports the concept[8] that a larval leaf beetle gland cell may possess a fundamental capacity for several biogenetic pathways. According to this concept one or several chemical pathways may be realized by a larva, depending on food plant chemistry and the need for certain physicochemical properties of the secretions: compounds from other pathways may then be absent or present only as trace constituents. It appears that sometimes chemical defence may be of limited advantage since larval representatives of two *Gonioctena* subgenera have obviously lost their defensive glands secondarily[18].

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Cardenolide biosynthesis in chrysomelid beetles

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Summary. Labeling experiments have shown that the chrysomelid beetle *Chrysolina coerulans* is able to biosynthesize its own defensive cardenolides from cholesterol, via a pathway involving a C_{21} intermediate, as in plants.
Key words. Biosynthesis; cardenolides; Coleoptera; chrysomelid beetles; [23-^{14}C]-cholesterol.

The presence of cardenolides has been reported in various insects of different orders including the aphid *Aphis nerii*[1], the bug *Oncopeltus fasciatus*[2], the grasshopper *Poekilocerus bufonius*[3] and the migratory butterfly *Danaus plexippus*[4]. All these insects feed on plants which have been shown to contain cardenolides and it is generally considered that the cardiac glycosides present in these insects are sequestered from their food plant[5].

Several insects of the family Chrysomelidae (Coleoptera) produce defensive secretions containing cardiac glycosides, thereby deriving their protection against predation[6-8]. In contrast to the aforementioned insects, all the cardenolide-containing chrysomelids studied until now feed on plants that are known to be devoid of cardiac glycosides (e.g., mint or rosemary). This suggests that these beetles are able to carry out the de novo biosynthesis of cardenolides. This hypothesis is supported by the observation[7] that adults of *Chrysolina polita* bred in the laboratory for four generations on *Mentha* × *villosa* still produce cardenolides. We wish to report here on experiments designed to supply a chemical basis for this hypothesis. Specimens of the species *Chrysolina coerulans* were fed with labeled cholesterol, a probable precursor of the cardenolides in chrysomelids. Indeed, it is well established that 1) in plants, the cardenolides arise from a C_{21} precursor resulting from the degradation of the phytosterol side chain[9,10]; and 2) phytophagous insects readily transform phytosterols into cholesterol in order to fulfill their metabolic requirements[11].

Leaves of *Mentha aquatica* were coated with 10 µl of a solution of [4-^{14}C]-cholesterol (total activity 0.1 mCi) in acetone and given as food to 45 adults of *C. coerulans*. The leaves were replaced either when decaying or when completely consumed, until administration of the precursor had been completed (approximately 2 leaves/insect/week). The secretions were collected by 'milking' on bits of filter paper. The filter papers were extracted three times with methanol and the extract evaporated in vacuo to yield 1.4 mg of dry material. It has been established in a previous work[7] that this secretion contains mainly six cardenolides, namely sarmentogenin **1**, periplogenin **2**, bipindogenin **3** and their corresponding xylosides **1a**, **2a** and **3a**.

The four major compounds present in the secretion, **1**, **1a**, **2a** and **3a** were purified by HPLC (C-18 reverse phase column, acetonitrile/water 1/3). Their specific activity was measured using a liquid scintillation counter (table 1).

The values reported in table 1 clearly show that [4-^{14}C]-cholesterol is incorporated into cardenolides, thus confirming our previous hypothesis of a de novo biosynthesis starting from cholesterol. These results prompted us to devise further incorporation