

Distribution of Toxins in Chrysomeline Leaf Beetles: Possible Taxonomic Inferences

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ABSTRACT

From a survey of the toxins produced by the pronotal and elytral defensive secretions in 114 chrysomeline species (20 genera *sensu* Daccordi 1994), three major groups of species are recognized which are considered as natural supra-generic taxa. These ensembles, however, do not perfectly fit existing classifications (e.g. Daccordi in Seeno and Wilcox 1982 or Daccordi 1994). Species secreting isoxazolinone glucosides esterified by nitropropanoic acid are considered as *Chrysomelina sensu stricto* which includes so far *Chrysomela*, *Linnaeidea*, *Plagioderia*, *Gastrophysa*, *Phaedon* (including *Hydrothassa*), *Prasocuris*, *Phaedonia* and *Phratora*. This supra-generic taxon is supported by larval characters, i.e. serial defensive glands, and by mtDNA phylogeny. The co-occurrence of serial glands in larvae and the adult toxins is so constant that the existence of serial glands in larvae is enough to qualify beetles as members of this taxon (e.g. *Mesoplatys* or *Gastrolina*). However, *Colaspidema* is excluded from it. Species secreting cardenolides or polyoxygenated steroids are considered as *Chrysolinina sensu stricto*, including so far *Chrysolina* (*sensu* Daccordi 1994), *Ambrostoma*, *Zygogramma*, *Cosmogamma*, *Calligrapha* and *Stilodes*. Polyoxygenated steroids are only secreted by members of three *Chrysolina* subgenera, *Sphaeromela*, *Hypericia* and *Chalcoidea*, which could be raised to distinct genera. Species secreting triterpene saponins are considered as *Doryphorina sensu stricto*, including so far *Platyphora*, *Leptinotarsa*, *Labidomera* and *Desmogramma*. While the classification proposed here is based on a restricted number of genera, we believe it may be useful in the building of a new and more natural classification of chrysomeline leaf beetles.

INTRODUCTION

Chrysomeline (Chrysomelini) suprageneric classification is notoriously difficult. For historical and pragmatic reasons, it is based on adult external morphology. Many of these characters may be homoplasious (i.e. due to convergent or parallel evolution), limiting their usefulness. Besides, gradual

changes between morphs seems to be frequent, and Mauro Daccordi with his wonderful Italian sense of humour, once suggested to the senior author of this paper that all Chrysomeline leaf beetles should belong to a single genus: *Chrysomela* Linnaeus. However, speciation normally is preceded by divergence of traits that can be used for building a natural classification of leaf beetles. Hence, leaf beetles experts explored additional attributes which may prove useful in classifying the Chrysomelini, e.g. morphology of immature stages (Bourdonné pers. com., Petitpierre and Juan 1994, Cox 1996 and references therein), caryotypes (Petitpierre 1988, Petitpierre *et al.* 1988), defensive toxins (Pasteels 1993), DNA sequences (Mardulyn *et al.* 1997, Hsiao and Pasteels 1999, Gomez-Zurita *et al.* 1999).

Our current research on the evolution of chemical defense in leaf beetles was not initially aimed at solving taxonomic problems. On the contrary, we hoped to be able to use current classifications for a better understanding of the evolution of leaf beetle defense. However, from our studies on chemical defense in Holarctic species, patterns emerged that were not consistent with current classifications (Pasteels 1993). Our recent studies of chemical defense in Neotropical leaf beetles reinforce this conclusion and disclose additional patterns that we will report here.

For the following reasons, only the distribution of three main classes of compounds, known to be released as defensive compounds from exocrine glands of adult chrysomelines, will be considered: isoxazolinone glucosides esterified by nitropropanoic acid, cardenolides and other steroids, and pentacyclic triterpene saponins. First, these compounds are easily detected either by mass spectrometry or thin-layer chromatography, even when secretions of only few individuals are available (these methods are fully described in papers reporting the identification of the beetle toxins, e.g., Daloze *et al.* 1991, 1995, Pasteels *et al.* 1982, 2001, Plasman *et al.* 2000a and b). Second, they are either synthesized by the beetles themselves or are derived from precursors widely distributed in the plant kingdom. Thus, oligophagy (as is the rule in all beetle taxa considered in this study) does not constrain beetle chemical defense. Third, they have yet to be found occurring together and hence appear mutually exclusive. Fourth, these compounds require very different metabolic mechanisms and biosynthetic pathways, so far unique to leaf beetles within the whole animal kingdom. Thus, convergence or parallel evolution of these traits appears extremely unlikely, suggesting that each class of toxin is a synapomorphy, defining a distinct clade.

It should be stressed here, that the occurrence of a toxin in a secretion is more informative than its absence, as a secondary loss of a synthetic ability is more likely to occur by a single mutation than the independent evolution of a multi-steps biosynthetic pathway requiring different enzymes. Several apparent exceptions were observed to the pattern, some species lacking the expected compound suggested by current classification. These exceptions will be discussed one by one. If the secretion contains compounds belonging to one or the other classes defining the other clades, we will consider this a strong indication of miss-classification. If the secretion does not contain any diagnostic compound, apparent contradiction with current classification will be discussed in the light of other available characters.

Besides these three classes of compounds, leaf beetles secretions contain a large diversity of other compounds (review in Pasteels *et al.* 1988a, 1994) that will not be discussed for one of the following two main reasons. First, their occurrence is narrowly constrained by the secondary chemistry of a small subset of host plants, and appears to be a secondary evolution in species which colonized those plants, e.g. sequestered pyrrolizidine alkaloids in some *Chrysolina* Motschulsky (*Chrysochloa* Hope, *Frigidorina* Khnhelt and *Intricatorina* Khnhelt) or *Platyphora* Gistel species (Pasteels *et al.* 1995, 2001). In these cases, parallel evolution cannot always be ruled out (Hsiao and Pasteels 1999). Second,

the presence of some compounds in the secretions cannot be detected without ambiguity by straightforward methods and they were not systematically searched for. Some of these compounds are possibly plesiomorphic characters as suggested by their tentative identification in unrelated taxa, e.g. some amino acid derivatives. Others appear as compounds yet observed in the secretions of a single species in admixture with more widespread toxins, e.g. chlorogenic acid in *Platyphora ligata* (StDI) or *N,N,N*-trimethylcadaverine in *Platyphora opima* (StDI) found together with triterpene saponins (Plasman *et al.* 2000a and b). In the future, some of these compounds could prove to be useful taxonomic characters when additional secretions are analyzed.

The two most recent and exhaustive classifications of chrysomeline leaf beetles will be used for discussing our results. Both were published by Daccordi (in Seeno and Wilcox 1982, and in Daccordi 1994). As different patterns were observed within the large genus *Chrysolina*, its subgeneric division will be taken into account. Although there are divergent opinions on the status of some taxa as *Chrysolina* subgenera or as distinct genera, Daccordi's 1994 list of subgenera will be used for the sake of consistency. Daccordi's 1994 classification mainly differs from his earlier one by the fusion of the subtribes Doryphorina Yuasa 1936 and Chrysolina Chen 1936 in a single subtribe Chrysolina, now subdivided into several groups of genera, by including the Gonioctenina Wilcox 1972 within the Paropsina Weise 1915, and by moving some genera from one subtribe to another.

MATERIALS AND METHODS

Chemical data were compiled from literature (reviews in Pasteels *et al.* 1988a and 1994, Daloze *et al.* 1995, Plasman *et al.* 2000a and b, 2001, Pasteels *et al.* 2001). Additional species were surveyed for the purpose of this study. Direct comparison by thin layer chromatography of unknown secretion extracts and those of related species, previously analyzed, identified nitropropanoic acid and isoxazolinone glucosides. Thin layer chromatography followed by spraying with Kedde's reagent revealed the presence of cardenolides in secretion extracts. Mass spectroscopy analyses of the raw extracts as described in Pasteels *et al.* (2001) were used for detecting triterpenes and cardenolides.

RESULTS

Isoxazolinone Glucosides Esterified by Nitropropanoic Acid (Fig. 1)

Isoxazolinone and nitropropanoic acid glucosides were reported from various legumes (e.g. *Astragalus* Linn., *Coronilla* Linn., *Indigophera* Linn., *Lathyrus* Linn., *Pisum* Linn., Harlow *et al.* 1975, Lambein *et al.* 1976, Gistine 1979, Ikegami *et al.* 1984, Majak *et al.* 1992). Nitropropanoic acid was also reported from fungi (Turner 1971), but from no insects other than leaf beetles. It is well known that nitropropanoic acid is a potent neurotoxin responsible for cattle poisoning (Coburn *et al.* 1975).

Leaf beetles do not obtain these compounds from their food plants, but synthesize them from aspartic acid (Randoux *et al.* 1991). In the defensive secretion, the major compounds are isoxazolinone glucosides esterified by one or two nitropropanoic acid residues (Pasteels *et al.* 1982, 1994). An esterase present in the secretion releases free nitropropanoic acid and the glucoside of isoxazolinone from the esterified glucosides illustrated in Fig.1 (Daloze, Kirk and Pasteels, unpublished data). In experiments with the ant *Myrmica rubra* Linn., the glucoside of isoxazolinone did not proved to be highly toxic, but to be an efficient ant deterrent (Pasteels *et al.* 1988b).

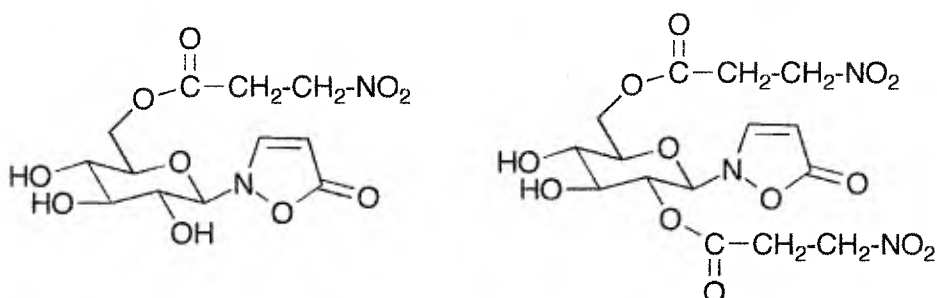


Fig. 1. Isoxazoline glucosides esterified by nitropropanoic acid.

Table 1. List of species secreting isoxazolinone glucosides esterified by nitropropanoic acid.

Chrysomela

aneicollis (Schaeffer), *collaris* L., *confluens* Rogers, *cuprea* F., *interrupta* F., *lapponica* L., *laurentia* Brown, *populi* L., *saliceti* (Weise), *schaefferi* Brown, *scripta* F., *tremulae* F., *vigintipunctata* (Scop.)

Gastrophysa

cyanea Melsheimer, *viridula* (De Geer)

Linaeidea

aenea (L.)

Phratora

americana (Schaeffer), *atrovirens* (Corn.), *laticollis* (Suffr.), *tibialis* (Suffr.), *vitellinae* (L.), *vulgatissima* (L.).

Plagioderia

versicolora (Laich.), *viridipennis* Stal

Phaedon

cochleariae (F.), (*Hydrothassa*) *marginella* (L.), (*Prasocuris*) *pbellandrii* (L.)

Pbaedonia

circumcincta (Sahlb.)

Leaf beetles secreting these compounds are listed in Table 1. Taxa known to produce them or not were replaced in both Daccordi's classifications in Tables 2A and 2B.

Cardenolides and Polyoxygenated Steroids (Figs. 2 and 3)

Cardenolides or cardiac glycosides are notorious plant toxins occurring as free genins, but more often as complex glycosides. The genins are steroids characterized by the presence of a five-membered lactone ring at C17. They were found in species of twelve plant families, above all in Asclepiadaceae and Apocynaceae. They inhibit the Na^+/K^+ pump in various tissues including cardiac tissue and some are used to treat heart disease. Besides, they are extremely bitter and emetic (see Malcom 1991 for a review of cardenolide distribution in plants and insects and of cardenolide biological activity).

Table 2. Distribution of genera with taxa secreting isoxazolinone glucosides esterified by nitropropanoic acid (in bold) according to Daccordi's 1982 (A) and 1994 (B) classifications.

| | |
|----------|---|
| A | Subtribe Doryphorina <i>Calligrapha</i> , <i>Cosmogramma</i> , <i>Desmogramma</i> , <i>Labidomera</i> , <i>Leptinotarsa</i> , <i>Platyphora</i> , <i>Stilodes</i> , <i>Zygogramma</i> . |
| | Subtribe Chrysolinina <i>Ambrostoma</i> , <i>Chrysolina</i> (<i>Allochrysolina</i> , <i>Allorina</i> , <i>Chalcoidea</i> , <i>Chrysobloa</i> , <i>Chrysolina</i> , <i>Chrysomorpha</i> , <i>Colaphodes</i> , <i>Erythrochrysa</i> , <i>Euchrysolina</i> , <i>Fastuolina</i> , <i>Frigidorina</i> , <i>Hypericia</i> , <i>Intricatorina</i> , <i>Maenadochrysa</i> , <i>Melasomoptera</i> , <i>Menthastriella</i> , <i>Oreina</i> , <i>Sphaeromela</i> , <i>Taeniochrysea</i> , <i>Virgulatorina</i>) |
| | Subtribe Chrysomelina <i>Chrysomela</i> , <i>Colaspidema</i> , <i>Linnaeidea</i> , <i>Gastrophysa</i> , <i>Phaedon</i> (including <i>Hydrothassa</i> and <i>Prasocuris</i>), <i>Phaenodonta</i> , <i>Plagioderia</i> . |
| | Subtribe Goniocetenina <i>Goniocetena</i> , <i>Phratora</i> . |
| B | Subtribe Paropsina <i>Goniocetena</i> |
| | Subtribe Chrysolinina <i>Ambrostoma</i> , <i>Chrysolina</i> (<i>Allochrysolina</i> , <i>Allorina</i> , <i>Chalcoidea</i> , <i>Chrysobloa</i> , <i>Chrysolina</i> , <i>Chrysomorpha</i> , <i>Colaphodes</i> , <i>Erythrochrysa</i> , <i>Euchrysolina</i> , <i>Fastuolina</i> , <i>Frigidorina</i> , <i>Hypericia</i> , <i>Intricatorina</i> , <i>Maenadochrysa</i> , <i>Melasomoptera</i> , <i>Menthastriella</i> , <i>Oreina</i> , <i>Sphaeromela</i> , <i>Taeniochrysea</i> , <i>Virgulatorina</i>) |
| | **** <i>Calligrapha</i> , <i>Zygogramma</i> |
| | **** <i>Stilodes</i> , <i>Leptinotarsa</i> , <i>Labidomera</i> |
| | **** <i>Stichosa</i> (= <i>Desmogramma</i>), <i>Platyphora</i> |
| | Subtribe Chrysomelina <i>Colaspidema</i> , <i>Gastrophysa</i> |
| | **** <i>Phaedon</i> (including <i>Hydrothassa</i> and <i>Prasocuris</i>) |
| | **** <i>Phratora</i> |
| | **** <i>Chrysomela</i> , <i>Linnaeidea</i> , <i>Plagioderia</i> |

Contrary to other insects which sequester cardenolides from plants (e.g. danaid butterflies and milkweed bugs, Brower and Glazier 1975, Scudder *et al.* 1986), leaf beetles are able to synthesize their own cardenolides from cholesterol (Van Oycke *et al.* 1987) and hence from ubiquitous plant sterols as all herbivorous insects must derive their sterols from plants. Cardenolides in leaf beetle secretions are diverse (34 fully identified so far and the structure of many more remain to be determined), differing either by their genin (six recognized so far) or by their sugar moieties (reviews in Pasteels *et al.* 1988a and 1994, Daloze *et al.* 1995). Leaf beetle cardenolides were found to deter ants at a concentration of 10^{-3} M and toxic at concentration of 10^{-2} M, much below the concentration in the secretions, about 10^{-1} M (Pasteels *et al.* 1988b).

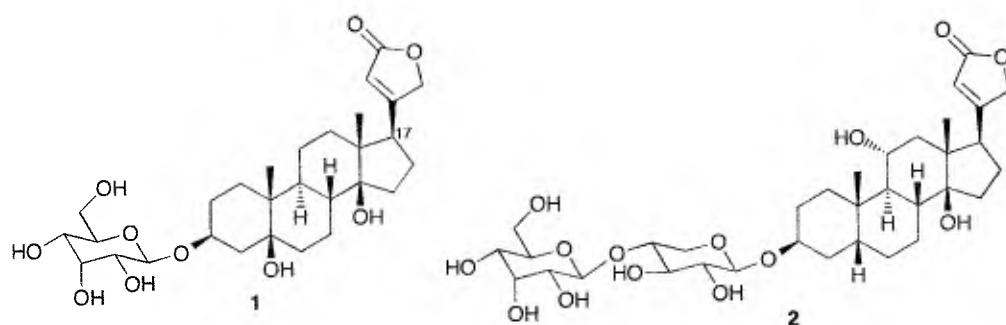


Fig. 2. Examples of cardenolides secreted by leaf beetles. (1) periplogenin-3-O-β-D-allopyranoside secreted by *Chrysolina* (*Oreina*) *gloriosa*; (2) sarmentogenin-3-O-β-D-xylopyranosyl-(1→4)-β-D-allopyranoside secreted by *Chrysolina* (*Allochrysolina*) *fuliginosa*.

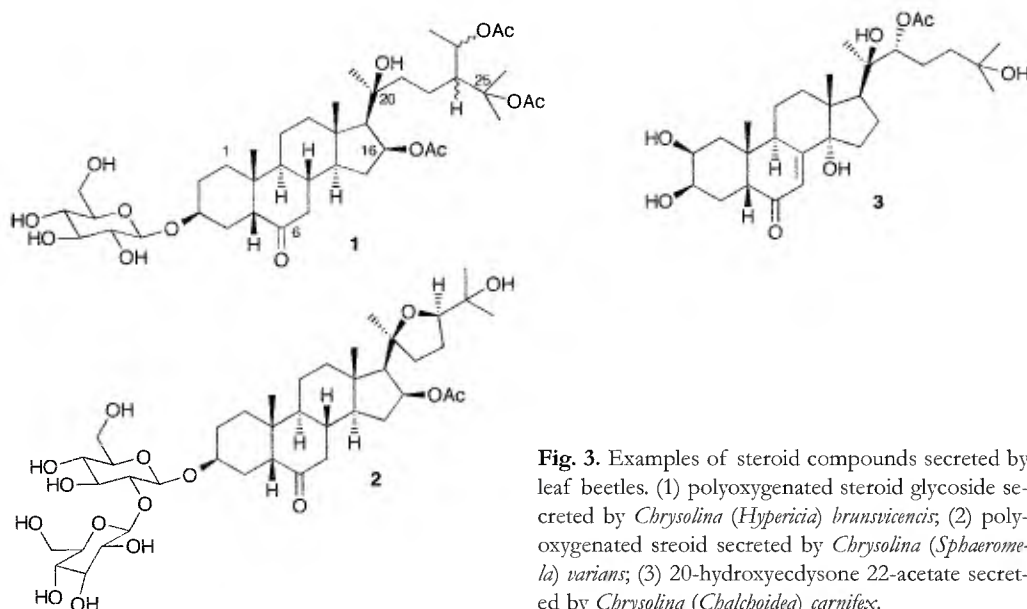


Fig. 3. Examples of steroid compounds secreted by leaf beetles. (1) polyoxygenated steroid glycoside secreted by *Chrysolina* (*Hypericia*) *brunsvicensis*; (2) polyoxygenated steroid secreted by *Chrysolina* (*Sphaerome-la*) *varians*; (3) 20-hydroxyecdysone 22-acetate secreted by *Chrysolina* (*Chalchoidea*) *carnifex*.

Leaf beetle polyoxygenated steroid glycosides are also diverse (13 identified so far, 12 different aglycones). All aglycones show a common oxidation pattern (at C-6, C-16, C-20, C-25) strongly suggesting a common evolutionary origin. Most compounds (the only exception is the ecdysteroid found in *Chrysolina carnifex* (Suffrian), see below) were isolated from beetles feeding on *Hypericum* Linn. which is devoid of these steroids (Daloze *et al.* 1985, 1991, Randoux *et al.* 1990). As far as we know, such steroids are unknown from other sources and must be synthesized by the leaf beetles from ubiquitous plant sterols as demonstrated for cardenolides. Recently, an ecdysteroid (obviously related to the above-mentioned polyoxygenated steroids) was identified in the secretion of *C. carnifex* (Braekman, Daloze and Pasteels, unpublished results). Whereas ecdysteroids are known as molting hormones in arthropods,

they were reported as defensive compounds in plants and pycnogonids (archaic marine arthropods) with a strong feeding deterrent activity (review in Tomaschko 1997). However, no ecdysteroid was reported from the food plant of *C. carnifex*, *Artemisia scoparia* Waldst. et Kit. (Asteraceae).

Species secreting cardenolides and polyoxygenated steroids are listed in Table 3 and this distribution is superimposed on Daccordi's classifications in Tables 4A and 4B.

Pentacyclic Triterpene Saponins (Fig. 4)

Again, these are well known plant toxins with haemolytic and cytotoxic activities, disrupting cell membranes (Mahato and Nandy 1991). Six different saponins were identified in leaf beetles secretions (Plasman *et al.* 2000 a and b, 2001). Only one of them was previously known from *Fagus sylvatica* Linn., not a food plant of chrysomeline beetles. All triterpene aglycones (five recognized so far, only differing by the position of one or two hydroxyl groups) are β -amyrin derivatives. Insects are unable

Table 3. List of species secreting steroids glycosides.

1. CARDENOLIDES

Ambrostoma

quadriimpressum Motschulsky

Calligrapha

alni Schaeffer, *argus* Stal, *multipunctata* bigsbyana (Kirby), *fulvipes* Stal, *philadelphica* (L.)

Chrysolina

(*Allochrysolina*) *lepida* (Olivier), *fuliginosa* (Olivier),

(*Allorina*) *bidentata* (Bontems), *coerulea* (Olivier), (*Chrysocloa*) *elongata* (Suffrian), *speciosissima* (Scopoli), (*Chrysolina*)

banksi (F.), *staphylea* (L.),

(*Chrysompha*) *cerealis* (L.), (*Colaphodes*) *haemoptera* (L.),

(*Erythrochrysa*) *polita* (L.), (*Euchrysolina*) *graminis* (L.), *virgata* (Motschulsky), (*Fastuolina*) *fastuosa* (Scopoli), (*Frigidorina*)

frigida (Weise),

(*Intricatorina*) *intricata* (Germar), (*Maenadochrysa*) *femoralis* (Olivier), (*Melasomoptera*) *lucida* (Olivier), *grossa* (F.),

(*Menthastriella*) *coerulans* (Scriba), *herbacea* (Duftschmid), *viridana* (Küster), (*Minckia*) *peregrina* (Herrich-Schäffer),

(*Oreina*) *alpestris variabilis* (Weise), *bifrons* (F.), *gloriosa* (F.), *speciosa* (L.),

viridis (Duftschmid), (*Taeniochrysea*) *americana* (L.),

(*Virgulatorina*) *virgulata* (Germar)

Cosmogramma

kimbergi Boheman

Stilodes

flavicans Stal, *fuscolineata* Stal

Zygogramma

sexvitata Stal, *signatipennis* Stal, *suturalis* (F.)

2. POLYOXYGENATED STEROID GLUCOSIDES OR ECDYSTEROID

Chrysolina

(*Chalcoidea*) *carnifex* (F.), (*Hypericia*) *brunsvicensis* (Gravenhorst),

hyperici (Förster), *geminata* (Paykull), *quadrigemina* (Suffrian),

(*Sphaeromela*) *varians* (Schaller)

Table 4. Distribution of genera with taxa secreting cardenolides (**bold**) or polyoxygenated steroid glucosides (**bold and underlined**) according to Daccordi's 1982 (A) and 1994 (B) classifications.

| | |
|-------------------------------|--|
| A Subtribe Doryphorina | |
| | <i>Calligrapha</i> , <i>Cosmogramma</i> , <i>Stichosa</i> (<i>Desmogramma</i>), <i>Labidomera</i> , <i>Leptinotarsa</i> , <i>Platyphora</i> , <i>Stilodes</i> , <i>Zygogramma</i> |
| Subtribe Chrysolinina | |
| | <i>Ambrostoma</i> , <i>Chrysolina</i> (<i>Allochrysolina</i> , <i>Allorina</i> , <i>Chalcoidea</i> , <i>Chrysobloa</i> , <i>Chrysolina</i> , <i>Chrysomorpha</i> , <i>Colaphodes</i> , <i>Erythrochrysa</i> , <i>Euchrysolina</i> , <i>Fastuolina</i> , <i>Frigidorina</i> , <i>Hypericia</i> , <i>Intricatorina</i> , <i>Maenadochrysa</i> , <i>Melasomoptera</i> , <i>Menthastriella</i> , <i>Oreina</i> , <i>Sphaeromela</i> , <i>Taeniochrysea</i> , <i>Virgulatorina</i>) |
| Subtribe Chrysomelina | |
| | <i>Chrysomela</i> , <i>Colaspidema</i> , <i>Linnaeidea</i> , <i>Gastrophysa</i> , <i>Phaedon</i> (including <i>Hydrothassa</i> and <i>Prasocuris</i>), <i>Phaedonia</i> , <i>Plagiodera</i> |
| Subtribe Gonioctenina | |
| | <i>Gonioctena</i> , <i>Phratora</i> . |
| B Subtribe Paropsina | |
| | <i>Gonioctena</i> |
| Subtribe Chrysolinina | |
| | <i>Ambrostoma</i> , <i>Chrysolina</i> (<i>Allochrysolina</i> , <i>Allorina</i> , <i>Chalcoidea</i> , <i>Chrysobloa</i> , <i>Chrysolina</i> , <i>Chrysomorpha</i> , <i>Colaphodes</i> , <i>Erythrochrysa</i> , <i>Euchrysolina</i> , <i>Fastuolina</i> , <i>Frigidorina</i> , <i>Hypericia</i> , <i>Intricatorina</i> , <i>Maenadochrysa</i> , <i>Melasomoptera</i> , <i>Menthastriella</i> , <i>Oreina</i> , <i>Sphaeromela</i> , <i>Taeniochrysea</i> , <i>Virgulatorina</i>). |
| | **** |
| | <i>Calligrapha</i>, <i>Zygogramma</i> |
| | **** |
| | <i>Stilodes</i> , <i>Leptinotarsa</i> , <i>Labidomera</i> |
| | **** |
| | <i>Stichosa</i> (<i>Desmogramma</i>), <i>Platyphora</i> |
| Subtribe Chrysomelina | |
| | <i>Colaspidema</i> , <i>Gastrophysa</i> |
| | **** |
| | <i>Phaedon</i> (including <i>Hydrothassa</i> and <i>Prasocuris</i>) |
| | **** |
| | <i>Phratora</i> |
| | **** |
| | <i>Chrysomela</i> , <i>Linnaeidea</i> , <i>Plagiodera</i> |

to biosynthesize pentacyclic triterpenes and it was recently demonstrated that the beetles sequestered β -amyrin from their food plant to synthesize their own saponins (Braekman, Daloze, Doods, Pasteels, Plasman, Termonia, unpublished results). Since β -amyrin is a common constituent of plant cuticular waxes (Gültz 1994), leaf beetles are little, if at all, constrained by their food plants to secrete these saponins.

Species secreting saponins are listed in Table 5 and saponin distribution in beetles superimposed on Daccordi's classifications in Tables 6 A and 6B.

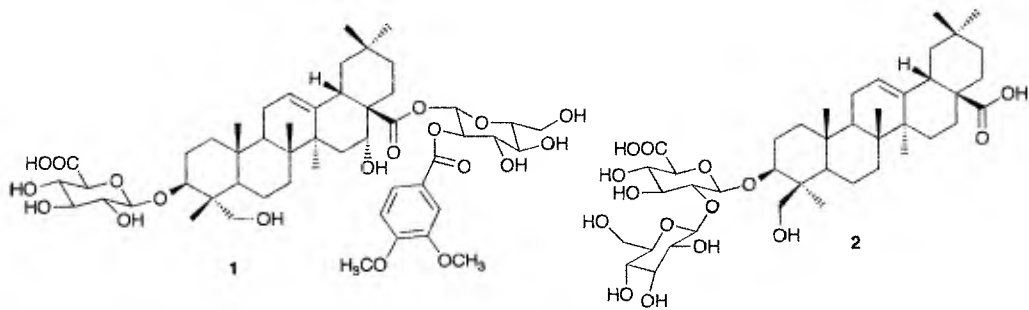


Fig. 4. Examples of pentacyclic triterpene saponins secreted by leaf beetles. (1) secreted by *Platyphora ligata*; (2) secreted by *Desmogramma subtropica*.

Table 5. List of species secreting triterpene saponins.

| |
|---|
| <i>Labidomera</i> <i>trimaculata</i> (F). |
| <i>Leptinotarsa</i> <i>bebrensi</i> Harold, <i>calceata</i> StDI, <i>baldemani</i> (Rogers), <i>lineolata</i> (StDI), close to <i>texana</i> Schaeffer, <i>undecimlineata</i> StDI |
| <i>Platyphora</i> <i>albovirens</i> (StDI), <i>amabilis</i> (Baly), <i>arangoi</i> (Steinh.), <i>bella</i> (Baly), <i>boucardi</i> (Jacoby), <i>decorata</i> (Jacoby), <i>eucosma</i> (StDI), <i>beliogenia</i> (Bechyné), <i>haroldi</i> (Baly), close to <i>iquitonensis</i> (Bechyné), <i>kollari</i> (StDI), <i>ligata</i> (StDI), <i>microspina</i> (Bechyné), <i>opima</i> (StDI), <i>panamensis</i> (Jacoby), <i>petulans</i> (StDI), close to <i>sahvini</i> (Baly), <i>spectabilis</i> (Baly), <i>vespertina</i> (Baly) |
| <i>Strichosa</i> (<i>Desmogramma</i>) <i>subtropica</i> Bechyné |

Table 6. Distribution of genera with taxa secreting triterpene saponins (**bold**) according to Daccordi's 1982 (A) and 1994 (B) classifications.

| |
|--|
| A Subtribe Doryphorina <i>Calligrapha</i> , <i>Cosmogramma</i> , <i>Strichosa</i> (<i>Desmogramma</i>) , <i>Labidomera</i> , <i>Leptinotarsa</i> , <i>Platyphora</i> , <i>Stilodes</i> , <i>Zygogramma</i> . |
| Subtribe Chrysolinina <i>Ambrostoma</i> , <i>Chrysolina</i> (<i>Allochrysolina</i> , <i>Allorina</i> , <i>Chalcoidea</i> , <i>Chrysochloa</i> , <i>Chrysolina</i> , <i>Chrysomorpha</i> , <i>Colaphodes</i> , <i>Erythrochrysa</i> , <i>Euchrysolina</i> , <i>Fastuolina</i> , <i>Frigidorina</i> , <i>Hypericia</i> , <i>Intricatorina</i> , <i>Maenadochrysa</i> , <i>Melasomoptera</i> , <i>Menthastriella</i> , <i>Oreina</i> , <i>Sphaeromela</i> , <i>Taeniochrysea</i> , <i>Virgulatorina</i>) |
| Subtribe Chrysomelina <i>Chrysomela</i> , <i>Colaspidea</i> , <i>Linaeidea</i> , <i>Gastrophysa</i> , <i>Phaedon</i> (including <i>Hydrothassa</i> and <i>Prasocuris</i>), <i>Phaedia</i> , <i>Plagioderia</i> |
| Subtribe Gonioctenina <i>Gonioctena</i> , <i>Phratora</i> . |

Table 6. Continued.

B Subtribe Paropsina*Gonioctena*

Subtribe Chrysolinina

Ambrostoma, *Chrysolina* (*Allochrysolina*, *Allorina*, *Chalcoidea*, *Chrysobloa*, *Chrysolina*, *Chrysomorpha*, *Colaphodes*, *Erythrochrysa*, *Euchrysolina*, *Fastuolina*, *Frigidorina*, *Hypericia*, *Intricatorina*, *Maenadochrysa*, *Melasomoptera*, *Menthastriella*, *Oreina*, *Sphaeromela*, *Taeniochrysea*, *Virgulatorina*)

Calligrapha, *Zygogramma*

Stilodes, ***Leptinotarsa***, ***Labidomera***

Stichosa* (*Desmogramma*)**, ***Platyphora

Subtribe Chrysomelina

Colaspidema, *Gastrophysa*

Phaedon (including *Hydrothassa* and *Prasocuris*)

Phratora

Chrysomela, *Linaeidea*, *Plagiadera***DISCUSSION**

Three clear-cut groups of chrysomeline beetles were recognized on the basis of the toxins they produce. For reasons argued in the Introduction, we suggest that they are natural taxa. However, they fit neither of Daccordi's classifications, although Daccordi's (1994) more recent classification seems in somewhat better agreement with our data than the former (in Seeno and Wilcox 1982). We do not intend to suggest an alternative classification to Daccordi's. We are, of course, aware that a classification cannot be built on a single class of characters, regardless of how convincing those characters may appear, and even more importantly before most if not all chrysomeline taxa could be studied. The following discussion is intended to provide a building block in the progressive construction of a natural classification of chrysomeline leaf beetles. Even if it could be overly simplistic at this stage of our survey of beetle toxins, we will tentatively assign these three groups to the Chrysomelina, Chrysolinina and Doryphorina *sensu stricto*, discussing the possible boundaries of these subtribes and their relationships, in the hope that this will stimulate further studies to enlarge the set of characters useful in the classification of these beetles.

Nitropropanoic acid and isoxazolinone secreting species or Chrysomelina sensu stricto

This seems the most convincing natural taxon in our study, as it has the support of independent morphological characters. All beetles secreting nitropropanoic acid and isoxazolinone have larvae characterized by nine serial pairs of thoracic and abdominal exsertile defensive glands. Reciprocally, all studied species whose larvae possess serial glands were found to produce these compounds (this represents a large proportion of existing taxa in the Holarctic Region, see Table 1). The agreement

between larval characters (serial defensive thoracic and abdominal defensive glands) and adult characters (nitropropanoic and isoxazolinone defensive compounds) looks so tight that we would not hesitate to include in this taxon, besides those listed in Table 1, taxa that are known to possess serial glands in the larval stage, but that were unavailable for studying the adult toxins, e.g. *Gastrolina* Baly or *Mesoplatus* Baly. Finally, a recent mtDNA phylogeny fully confirms that the Chrysomelina as here defined is a monophyletic clade (Termonia *et al.* 2001).

Members of this Chrysomelina *sensu stricto* were placed in two different subtribes in Daccordi's 1982 classification (Table 2A), however, the 1994 classification fits better our data as it now includes *Pbratora* Chevrolat, but not *Gonioclena* Chevrolat, within the Chrysomelina (Table 2B). Indeed, in contrast to *Pbratora* larvae, those of *Gonioclena* lack the serial glands diagnostic of the Chrysomelina *sensu stricto*. Also, our preliminary survey of the adult defensive secretions in ten species of *Gonioclena* belonging to 5 subgenera revealed that their secretions contain amino acid derivatives, but not nitropropanoic acid or isoxazoline glucosides (Brackman, Daloze, Mardulyn and Pasteels, unpublished data). However, the Chrysomelina *sensu* Daccordi 1994 includes taxa that do not meet our criteria for belonging to the Chrysomelina *sensu stricto*, e.g. *Colaspidema atra* Olivier (Pasteels, 1993). Molecular phylogenies published by Hsiao (1994, see his Fig. 4) confirms that *C. atra* belongs to a lineage very distantly related to that of the Chrysomelina *sensu stricto*, strongly suggesting that the Chrysomelina *sensu* Daccordi 1994 is still an unnatural ensemble. Unfortunately, we have no alternative to offer for classifying *C. atra* except as Chrysomelini *incertae sedis* and we cannot define at this stage the exact boundaries of the Chrysomelina *sensu stricto*.

Cardenolides and polyoxygenated steroids secreting species or Chrysolina sensu stricto

Although the polyoxygenated steroids (including an ecdysteroid) are quite distinct compounds from the cardenolides, and are only secreted by members of three *Chrysolina* subgenera, we are lumping together all species secreting steroids in a single suprageneric taxon. These species share the ability to use plant sterols for synthesizing toxins and this particularity makes them very distinctive from the other two groups recognized here. Excluding the species secreting polyoxygenated steroids from the cardenolide secreting species is untenable considering their undisputed affinities with the other *Chrysolina*. However, polyoxygenated steroids seem distinct enough from cardenolides that species secreting them could deserve generic rather than subgeneric status. This is strengthened by other evidence. *Hypericia* Bedel and *Sphaeromela* Bedel species feed on *Hypericum*, an unusual food plant for *Chrysolina*, and adult morphological characters isolate them from the other *Chrysolina* (Bourdonné and Doguet 1991). Also, phylogenies based on mtDNA sequence confirm that *Hypericia* and *Sphaeromela* form a monophyletic lineage (Hsiao and Pasteels 1999). *Chrysolina carnifex*, which secrete an ecdysteroid, belongs to the subgenus, *Chalcoidea* Motschulsky, that Jean-Claude Bourdonné is in the process of raising to the generic level on the basis of larval morphological characters (Bourdonné pers. com.). Our results point to an unsuspected affinity between *Hypericia*, *Sphaeromela* and *Chalcoidea*. Additional species must be studied to confirm this, and new molecular phylogenies will be very instructive in this respect.

Thus far, only one *Chrysolina* species, *Chrysolina (Chrysochloa) cavaliae* (Schränk) does not secrete steroids. However, this is a recent secondary evolution from synthesis of cardenolides to sequestration of plant pyrrolizidine alkaloids. Other *Chrysochloa* species synthesize both cardenolides and sequester pyrrolizidine alkaloids (Pasteels *et al.* 1995).

The Chrysolinina *sensu stricto* as here recognized includes taxa classified by Daccordi (1982) in the Chrysolinina, but also in the Doryphorina (Table 4A). In 1994, Daccordi fused the two subtribes

into the Chrysolinina. This change is only partly consistent with our data. *Zygogramma* Chevrolat, *Stilodes* Chevrolat, *Calligrapha* Chevrolat and *Cosmogramma* Erichson should indeed be placed among the Chrysolinina, but not necessarily *Leptinotarsa* Stål, *Labidomera* Chevrolat, *Platyphora* and *Desmogramma* Erichson (see below). Also, the distribution of cardenolides and triterpenes among taxa does not fit Daccordi's groups of genera of his 1994 Chrysomelina (Tables 4B and 6B).

Species secreting triterpene saponins or Doryphorina sensu stricto

Thus far triterpene saponins are known from members of four genera, all previously classified by Daccordi (1982) within the Doryphorina (Tables 5 and 6A). Secretion of saponins requires the use of very different plant precursors and biosynthetic pathways than the secretion of toxins in the other genera, justifying their grouping in a different suprageneric taxon. Also, Hsiao's phylogenies based on mtDNA sequences demonstrated that at least three of these genera, *Platyphora*, *Labidomera*, and *Leptinotarsa* form a monophyletic lineage distinct from the *Calligrapha* and *Zygogramma* lineage, now placed in the Chrysolinina *sensu stricto* (see above). It remains unclear whether the Doryphorina *sensu stricto* is a sister clade of the Chrysolinina *sensu stricto* or a subset of it (see Fig 4. in Hsiao 1994). More research is needed to determine the hierarchy and boundaries of the suprageneric taxa here recognized.

Not all *Platyphora* and *Leptinotarsa* species secrete saponins. We could not detect saponins in the secretions of *P. sphaerica* (Jacoby), *L. decemlineata* Say, *L. tlascalana* Stål, and *L. typographica* Jacoby. None of these species, however, secrete toxins that characterize the other two groups. We consider this absence of saponins as a secondary loss, easier to evolve than independent acquisition of a synthetic ability (see Introduction), even if we cannot explain why the capacity of secreting saponins could have been lost in these species.

FINAL COMMENTS

Three clear-cut groups of Chrysomeline genera are recognized by the toxins they secrete, but to suggest a classification on this basis would be, at best, very preliminary and tentative. First, as ironically stated by Mauro Daccordi in a private discussion with the senior author of this paper, the categories are possibly clear-cut only because a restricted number of species were studied, but when more species will be added to the study, the picture could become completely blurred. Second, it would be, of course, circular reasoning to only use the observed distribution of beetle toxins to build a classification that is then used to explain the present distribution of toxins among taxa and the evolution of chemical defense in leaf beetles.

We still lack a natural classification of chrysomeline leaf beetles that could allow us to interpret our data in an evolutionary perspective. Our goal was to stimulate taxonomists to reconsider chrysomeline classification by all possible means to reach a more natural classification. We believe that chemical secretion data are useful, but the major advances will come from comparisons of DNA sequences. Work in this direction is in progress in our laboratories.

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LITERATURE CITED

- Bourdonné, J.-C. and S. Doguet. 1991. Données sur la biosystématique des *Chrysolina* L.S. (Coleoptera: Chrysomelidae: Chrysomelinae). *Annales de la Société Entomologique de France* (NS) 27:29-64.
- Brower, L. P. and S. C. Glazier. 1975. Localization of heart poisons in the monarch butterfly. *Science* 188:19-25.
- Coburn, W. M., F. R. Stermitz and R. D. Thomas. 1975. Nitro compounds in *Astragalus* species. *Phytochemistry* 14:2306-2308.
- Daccordi, M. 1982. Chrysomelinae. In: Seeno, T. N. and J. A. Wilcox. 1982. Leaf beetle genera (Coleoptera: Chrysomelidae). *Entomography* 1:75-95.
- Daccordi, M. 1994. Notes for phylogenetic study of Chrysomelinae, with descriptions of new taxa and a list of all the known genera (Coleoptera: Chrysomelidae, Chrysomelinae), pp. 60-84. In: D. G. Furth (Ed.), *Proceedings of the Third International Symposium on the Chrysomelidae*. Beijing, 1992. Backhuys, Leiden.
- Daloze, D., Braekman, J.-C. and J. M. Pasteels. 1985. New polyoxygenated steroidal glucosides from *Chrysolina hyperici*. *Tetrahedron Letters* 26:2311-2314.
- Daloze, D., J.-C. Braekman, A. Delbrassine and J. M. Pasteels. 1991. Polyoxygenated steroid sophorosides from the defence glands of *Chrysolina quadrigemina* (Coleoptera; Chrysomelidae). *Journal of Natural Products* 54:1553-1557.
- Daloze, D., F. Broeders, J.-C. Braekman, J. Araujo and J. M. Pasteels. 1995. New cardiac glycosides containing 2-deoxyhexoses from the defensive secretion of adult *Chrysolina banksi* (Coleoptera: Chrysomelidae). *Biochemical Systematics and Ecology* 23:113-119.
- Gistine, D. L. 1979. Aliphatic nitro compounds in crownvetch; a review. *Crop Science* 19:197-203.
- Gomez-Zurita, J., C. F. Garin, C. Juan, and E. Petitpierre. 1999. Mitochondrial 16s rDNA sequences and their use as phylogenetic markers in leaf-beetles with special reference to the subfamily Chrysomelinae, pp. 25-38. In: M. L. Cox (Ed.), *Advances in Chrysomelidae Biology 1*. Backhuys Publishers, Leiden.
- Gülz, P. G. 1994. Epicuticular leaf waxes in the evolution of the Plant Kingdom. *Journal of Plant Physiology* 143:453-464.
- Harlow, M. C., F. R. Stermitz and R. D. Thomas. 1975. Isolation of nitro compounds from *Astragalus* species. *Phytochemistry* 14:1421-1423.
- Hsiao, T. H. 1994. Molecular techniques for studying systematics and phylogeny of Chrysomelidae, pp. 237-248. In: P. H. Jolivet, M. L. Cox and E. Petitpierre (Eds.), *Novel Aspects of the Biology of Chrysomelidae*. Kluwer, Dordrecht.
- Hsiao, T. H. and J. M. Pasteels. 1999. Evolution of host-plant affiliation and chemical defense in *Chrysolina-Oreina* leaf beetles as revealed by mtDNA phylogenies, pp. 321-342. In: M. L. Cox (Ed.), *Advances in Chrysomelidae Biology 1*. Backhuys Publishers, Leiden.
- Ikegami, F., F. Lambein, Y. H. Kuo and I. Murakoshi. 1984. Isoxazolinon-5-one derivatives in *Lathyrus odoratus* during development and growth. *Phytochemistry* 23:567-1569.
- Lafont, R., A. Bouthier and I. D. Wilson. 1991. Phytoecdysteroids: Structures, occurrence, biosynthesis and possible ecological significance, pp. 197-214. In: I. Hrdy (Ed), *Insect Chemical Ecology*. Academia, Praha.

- Mahato, S. B. and A. K. Nandy. 1991. Triterpenoid saponins discovered between 1987 and 1989. *Phytochemistry* 30:1357-1390.
- Majak, W., M. Benn, D. McEwan and M. A. Pass. 1992. Three nitropropanoyl esters of glucose from *Indigofera linnaei*. *Phytochemistry* 31:2393-2395.
- Malcom, S. B. 1991. Cardenolide-mediated interactions between plants and herbivores, pp. 251-296. *In*: G. A. Rosenthal and M. R. Berenbaum (Eds.), *Herbivores: Their Interactions with Secondary Plant Metabolites*. Second Edition. Vol 1. The Chemical Participants. Academic Press, San Diego.
- Mardulyn, P., M. C. Milinkovitch and J. M. Pasteels. 1997. Phylogenetic analyses of DNA and allozyme data suggest that *Gonioctena* leaf beetles (Coleoptera, Chrysomelidae) experienced convergent evolution in their history of host-plant family shifts. *Systematic Biology* 46:722-747.
- Pasteels, J. M. 1993. The value of defensive compounds as taxonomic characters in the classification of leaf beetles. *Biochemical Systematics and Ecology* 21:135-142.
- Pasteels, J. M., J.-C. Braekmanand, D. Daloze and R. Ottinger. 1982. Chemical defense in chrysomelid larvae and adults. *Tetrahedron* 38:1891-1897.
- Pasteels, J. M., Braekman, J.-C. and D. Daloze. 1988a. Chemical defense in the Chrysomelidae, pp. 233-252. *In*: P. Jolivet, E. Petitpierre and T. H. Hsiao (Eds.), *Biology of Chrysomelidae*. Kluwer, Dordrecht.
- Pasteels, J. M., M. Rowell-Rahier and M. J. Raupp. 1988b. Plant-derived defense in chrysomelid beetles, pp. 235-272. *In*: P. Barbosa and D. K. Letourneau (Eds.), *Novel Aspects of Insect-Plant Interactions*. Wiley, New York.
- Pasteels, J. M., M. Rowell-Rahier, J.-C. Braekman and D. Daloze. 1994. Chemical defense of adult leaf beetles updated, pp. 289-301. *In*: P. H. Jolivet, M. L. Cox and E. Petitpierre (Eds.), *Novel Aspects of the Biology of Chrysomelidae*. Kluwer, Dordrecht.
- Pasteels, J. M., S. Dobler, M. Rowell-Rahier, A. Ehmke and T. Hartmann. 1995. Distribution of autogenous and host-derived chemical defenses in *Oreina* leaf beetles (Coleoptera, Chrysomelidae). *Journal of Chemical Ecology* 21:1163-1179.
- Pasteels, J. M., A. Termonia, D. M. Windsor, L. Witte, C. Theuring and T. Hartmann. 2001. Pyrrolizidine alkaloids and pentacyclic triterpene saponins in the defensive secretions of *Platyphora* leaf beetles. *Chemoecology* 11:113-120.
- Petitpierre, E. 1988. Cytogenetics, cytotaxonomy and genetics of Chrysomelidae, pp. 131-160. *In*: P. Jolivet, E. Petitpierre and T. H. Hsiao (Eds.), *Biology of Chrysomelidae*. Kluwer, Dordrecht.
- Petitpierre, E. and C. Juan. 1994. Genome size, chromosomes and egg-chorion ultrastructure in the evolution of Chrysomelinae, pp. 213-225. *In*: P. H. Jolivet, M. L. Cox and E. Petitpierre (Eds.), *Novel Aspects of the Biology of Chrysomelidae*. Kluwer, Dordrecht.
- Petitpierre, E., C. Segarra, J. S. Yadav and N. Virkki. 1988. Chromosome numbers and meioformulae of Chrysomelidae, pp. 161-186. *In*: P. Jolivet, E. Petitpierre and T. H. Hsiao (Eds.), *Biology of Chrysomelidae*. Kluwer, Dordrecht.
- Plasman, V., J.-C. Braekman, D. Daloze, M. Luhmer, D. M. Windsor and J. M. Pasteels. 2000a. Triterpene saponins in the defensive secretion of a chrysomelid beetle, *Platyphora ligata*. *Journal of Natural Products* 63:646-649.
- Plasman, V., J.-C. Braekman, D. Daloze, D. M. Windsor and J. M. Pasteels. 2000b. Triterpene saponins, quaternary ammonium compounds, phosphatidyl cholines, and amino acids in the pronotal and elytral secretions of *Platyphora opima* and *Desmogramma subtropica*. *Journal of Natural Products* 63:1261-1264.
- Plasman, V., M. Plehiers, J.-C. Braekman, D. Daloze, J.-C. de Biseau and J. M. Pasteels. 2001. Chemical defense in *Platyphora kollari* Baly and *Leptinotarsa bebbrensi* Harold (Coleoptera; Chrysomelidae). Hypothesis on the origin and evolution of leaf beetles toxins. 2001. *Chemoecology* 11:107-112.

- Randoux, T., J.-C. Braekman, D. Daloze and J. M. Pasteels. 1990. New polyoxygenated steroid glycosides from the defence glands of several species of Chrysolinina beetles (Coleoptera: Chrysomelidae). *Tetrahedron* 46:3879-3888.
- Randoux, T., J.-C. Braekman, D. Daloze and J. M. Pasteels. 1991. De novo biosynthesis of Δ^3 -isoxazolin-5-one and 3-nitropropanoic acid derivatives in *Chrysomela tremulae*. *Naturwissenschaften* 78:313-314.
- Scudder, G. G. E., L. V. Moore and M. B. Isman. 1986. Sequestration of cardenolides in *Oncopeltus fasciatus*. Morphological and physiological adaptations. *Journal of Chemical Ecology* 12:1171-1187.
- Tomaschko, K.-H. 1997. Ecdysteroids in pycnogonids: hormones and interspecific allelochemicals, pp.171-188. *In*: K. Dettner, G. Bauer and W. Voelki (Eds.), *Vertical Food Web Interactions*, Ecological Studies, vol. 30. Springer- Verlag, Berlin.
- Termonia, A., T. H. Hsiao, J. M. Pasteels and M. C. Milinkovitch. 2001. Feeding specialization and host-derived chemical defense in Chrysomeline leaf beetles did not lead to an evolutionary dead end. *Proceedings of the National Academy of Sciences* 98:3909-3914.
- Turner, W. B. 1971. Fungal Metabolites, pp. 303-304. Academic Press, London.
- Van Oycke, S., J.-C. Braekman, D. Daloze and J. M. Pasteels. 1987. Cardenolide biosynthesis in chrysomelid beetles. *Experientia* 43:460-462.