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Ladybird defence alkaloids: structural, chemotaxonomic and biosynthetic aspects (Col.: Coccinellidae)

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Summary. The defensive mechanisms which protect ladybird beetles (Coccinellidae) against predators are reviewed. Besides behavioural mechanisms, such as thanatosis and reflex bleeding, chemical defence mechanisms are playing a prevalent role. Indeed, ladybirds are protected not only by their smell, but also by repulsive alkaloids, most of which are considered to be of autogenous origin. In a few cases, dietarily-acquired substances are also involved. Particular emphasis is laid on the repellent alkaloids which are contained in the haemolymph of many species. The structures of 34 nitrogen-containing compounds isolated so far are presented, and their distribution within the family is discussed in the light of the most widely accepted classification of these beetles. To conclude, the mode of release of the alkaloids, their variation through the life cycle and their repellent and toxic properties are discussed, as well as the few biosynthetic data yet available.

Key words. chemical defence – sequestration – reflex bleeding – alkaloids – chemotaxonomy – biosynthesis – repellency – toxicity – Coleoptera – Coccinellidae

Introduction

The family Coccinellidae (ladybird beetles) comprises over 5,200 species worldwide. It shows a diversity of biological habits and food preferences. Most of these beetles are carnivorous, showing a voracious appetite for aphids, coccids and mites, but some are phytophagous and others mycophagous (Hodek 1973; Majerus 1994). The predaceous species have played an important role in the development of biological control methods of agricultural pests. For example, in the 1880s, several ladybird species were imported to California to control the scale insect *Icerya purchasi* which was threatening to destroy the citrus orchards (De Bach 1964).

Many ladybird species are characterized by beautiful red-orange colours which render them highly conspicuous to vertebrate predators, especially when they form dense aggregations during dormancy. Early studies have demonstrated that coccinellids were unacceptable to several predators of insects (Cuénot 1896; Hollande 1911; Frazer & Rothschild 1960), which is not unexpected as these beetles are strongly smelling, bad tasting and, in some cases, poisonous. The bright colours and distinct smell are warning the potential vertebrate predator that these beetles possess chemical defences. The most thoroughly studied defence mechanism of coccinellids is the so-called "reflex bleeding": when they are molested or disturbed, they fall into thanatosis and exude a yellowish fluid from the tibiofemoral articulations (Cuénot 1896; Hollande 1911, 1926; Happ & Eisner 1961). The bitterness and toxicity of the emitted fluid, together with the characteristic odour of these insects, have been regarded as a protection against insect or vertebrate predators.

As early as 1960, Rothschild put forward her theory of odour-based Müllerian mimicry among insects (Rothschild 1961). This theory got a sound chemical basis thirty years later when it was found that the smell of 45 aposematic insect species from 4 orders was due, at least in part, to 2-methoxy-3-alkylpyrazines (Moore & Brown 1981; Moore *et al.* 1990). Furthermore, these authors concluded that whereas most of the dull coloured ladybirds were virtually devoid of pyrazines, all of the aposematic, psyllid- or aphid-feeding species contained 2-methoxy-3-alkylpyrazines. Thus, the worldwide distribution of the colour red as an alerting signal is probably paralleled by pyrazines at the olfactory level (Moore *et al.* 1990).

Much research has been carried out on the chemical defence systems of ladybirds. Thanks to the pioneering studies of Tursch and coworkers in the early 70's, it is now well established that many coccinellids owe their protection to the presence of repellent and, in some cases, toxic alkaloids, some of which, at least, are autogenously produced (Tursch *et al.* 1976; Ayer & Browne 1977; Jones & Blum 1983). Thus, in this group, sequestration of dietary compounds for protective purposes seems to be much rarer.

In this review, we will first discuss the few reports of sequestration, then, we will focus on the defensive alkaloids that have been isolated so far from the Coccinellidae. We will discuss their structures and their distribution in light of the currently accepted taxonomy of the family (Sasaji 1968). In addition, informations on both the mode of release and the biosynthetic origin of the alkaloids will be reviewed, as well as possible differences between the different developmental stages. To conclude, the defensive efficiency of these compounds will be discussed. The impressive synthetic work that has already been performed on these alkaloids will not be reviewed here.

Sequestration of defensive chemicals from the prey

Many cases are known of insects acquiring defensive chemicals from their host plant (see refs in Rosenthal & Berenbaum 1991). This situation, however, is much less documented in the case of predaceous insects, which is not unexpected taking into account the wealth of secondary metabolites produced by the plant kingdom. Coccinellid beetles which are mostly predaceous follow this rule. Nevertheless, three interesting examples of sequestration of toxic or repellent compounds by carnivorous ladybird beetles have been reported up to now. *Coccinella 11-punctata* sequesters unidentified cardiac glycosides when feeding on the oleander aphid, *Aphis nerii* (Rothschild *et al.* 1973), which is itself a well-known cardiac glycoside sequestrator (Rothschild *et al.* 1970). In contrast, freshly hatched *C. 7-punctata* which has fed as larvae on this aphid were devoid of cardiac glycosides (Rothschild *et al.* 1973). On the other hand, *C. 7-punctata* feeding on *Aphis jacobaeae* colonizing *Senecio jacobaea* or *S. inaequidens* was found to sequester considerable amounts of pyrrolizidine alkaloids (up to 4.9 mg/g of fresh weight). Thus the sequestered pyrrolizidine alkaloids may reach 10 to almost 50% of the endogenous alkaloids. It is also interesting to note that, in both the aphids and the ladybirds, the sequestered alkaloids are the tertiary bases and not the N-oxides which are exclusively present in the plants (Witte *et al.* 1990). The results described above are fascinating examples of transmission of defensive chemicals at a tritrophic level, from a plant to a herbivorous, to a carnivorous species, each species apparently producing or sequestering these compounds for its own benefit. The last example in this section illustrates the transmission of an insect-produced repellent, namely carminic acid produced by cochineal bugs (Homoptera: Coccidae), to the larva of a predaceous ladybird beetle, *Hyperaspis trifurcata*. This larva was shown to reflex bleed when disturbed, and its haemolymph contained carminic acid at concentrations (1.3%) which are in a range known to be highly deterrent to ants (Eisner *et al.* 1994). It is not known if the larvae and the adults of *H. trifurcata* also contain alkaloids, which, as discussed below, are the most common defensive compounds of the family.

To our knowledge, only three phytophagous ladybird beetles have been studied till now for their alkaloid content, namely *Epilachna varivestis* (Attygalle *et al.* 1993a; Proksch *et al.* 1993), *E. guttatopustulata* (unpubl.) and *Subcoccinella 24-punctata* (Wang *et al.* 1996). In the three cases, the alkaloids isolated (see next section) do not appear to derive from the host plant.

Structures and distribution of alkaloids

The most widely accepted classification of the Coccinellidae is that of Sasaji (1968), who divided the family into six subfamilies: Sticholotinae, Scymninae, Chilocorinae, Coccidulinae, Coccinellinae and Epilachninae,

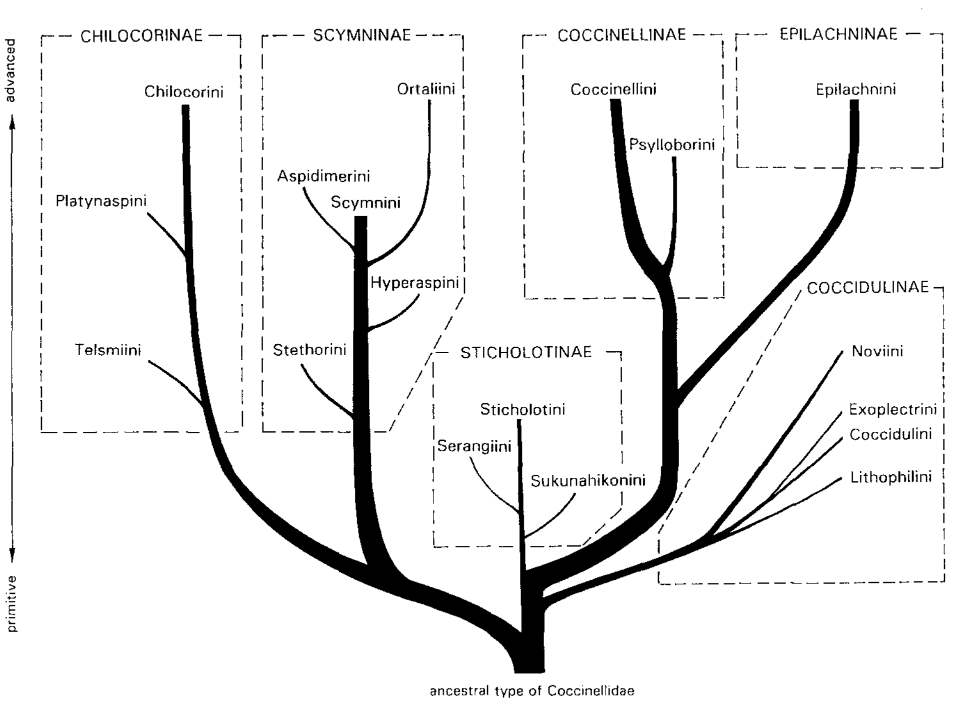


Fig. 1 Classification of Coccinellidae after Sasaji (1968)

Table 1 Distribution of alkaloids in Coccinellidae

	Alkaloids	References
Coccinellinae		
Coccinellini		
Coccinella 7-punctata	1, 2	Tursch *et al.* 1971a,b
C. 5-punctata	1, 2	Tursch *et al.* 1975
C. 11-punctata	2	Tursch *et al.* 1975
C. 14-punctata	1, 2	Tursch *et al.* 1975
C. californica	2	Tursch *et al.* 1975
C. transversoguttata	1, 2	Ayer *et al.* 1976
Coccinula 14-punctata	1, 2	Tursch *et al.* 1975
Cheilomenes propinqua	1, 2	Tursch *et al.* 1975
Micraspis 16-punctata	1, 3	Tursch *et al.* 1975; unpubl.
Coleomegilla maculata	1	Henson *et al.* 1975
Hippodamia convergens	3, 4, 9, 10	Tursch *et al.* 1974; Braconnier *et al.* 1985a
H. caseyi	3, 4, 7, 8	Ayer *et al.* 1976
Anisosticta 19-punctata	3	Tursch *et al.* 1975
Myrrha 18-guttata	5	Tursch *et al.* 1975
Propylaea 14-punctata	6A, 6B	Tursch *et al.* 1972
Harmonia 4-punctata	9	Braconnier *et al.* 1985a
Harmonia leis conformis	9	Braconnier *et al.* 1985a
Adonia variegata	9	Braconnier *et al.* 1985a
Semiadalia 11-notata	9	Braconnier *et al.* 1985a
Adalia bipunctata	11, 12	Tursch *et al.* 1973; Lognay *et al.* 1996
A. 10-punctata	11, 12	Tursch *et al.* 1973; Lognay *et al.* 1996
Chilocorinae		
Chilocorini		
Exochomus 4-pustulatus	13	Timmermans *et al.* 1992
Chilocorus cacti	14, 15	McCormick *et al.* 1994; Shi *et al.* 1995
Epilachninae		
Epilachnini		
Epilachna varivestis	16, 17–19, 21–33	Eisner *et al.* 1986; Attygalle *et al.* 1993a,b; Proksch *et al.* 1993
E. guttatopustulata	23	unpubl.
Subcoccinella 24-punctata	34	Wang *et al.* 1996
Scymninae		
Scymnini		
Cryptolaemus montrouzieri	16, 19, 20	Brown & Moore 1982

which are further divided into tribes. This classification scheme which, more than the previous ones, takes into account the evolutionary relationships of the different groups in the family is shown in Fig. 1.

In this review, we will limit ourselves to coccinellid species whose alkaloids have been fully characterized. The distribution of known alkaloids within the Coccinellidae is reported in Table 1. It is striking that, of all coccinellid tribes recorded by Sasaji (1968), alkaloids have been found in representatives of only four tribes: Coccinellini, Chilocorini, Epilachnini, and Scymnini. Moreover, only the tribe Coccinellini has been studied in some depth, with 21 alkaloid-producing species reported so far. In contrast, the three other tribes are only represented by one to three species (Table 1).

The tribe Coccinellini (subfamily Coccinellinae) is one of the largest groups. It is widely distributed and accordingly has been the most studied from a chemical point of view. All the alkaloids reported so far from this tribe are shown in Fig. 2. The first alkaloid ever to have been isolated from a coccinellid beetle is the tricyclic *N*-oxide coccinelline [2], which was found in the European *Coccinella 7-punctata*, accompanied by the corresponding free base, precoccinelline [1] (Fig. 2) (Tursch *et al.* 1971a,b). The structure and relative configuration of coccinelline hydrochloride, which is achiral due to the presence of a plane of symmetry, was solved by an X-ray diffraction study (Karlsson & Losman 1972). Compounds [1] and [2] were the first representatives of a new family of alkaloids based on the 2-methylperhydro-9b-azaphenalene (or hydropyrido[2,1,6-de]quinolizine) ring system. These alkaloids seem to be widely distributed in the genus *Coccinella* (Pasteels *et al.* 1973; Tursch *et al.* 1975; Ayer *et al.* 1976), but they have also been found in other Coccinellini genera, such as *Coccinula*, *Cheilomenes* and *Micraspis* (Tursch *et al.* 1975), and *Coleomegilla* (Henson *et al.* 1975) (Table 1). It is puzzling to observe that whilst some species (*e.g. C. 7-punctata*) always produce both precoccinelline [1] and its N-oxide [2], others seem to produce either [1] or [2] (*C. 11-punctata*, *C. californica*). The biological significance of this apparent specificity however remains obscure.

Hippodamine [3] and convergine [4] (Fig. 2) which are stereoisomers of [1] and [2] respectively were subsequently found in the American ladybird *Hippodamia convergens*. The structure and absolute configuration of convergine hydrochloride were established by an X-ray diffraction analysis (Tursch *et al.* 1974). These latter compounds are chiral, but the optical rotation of con-

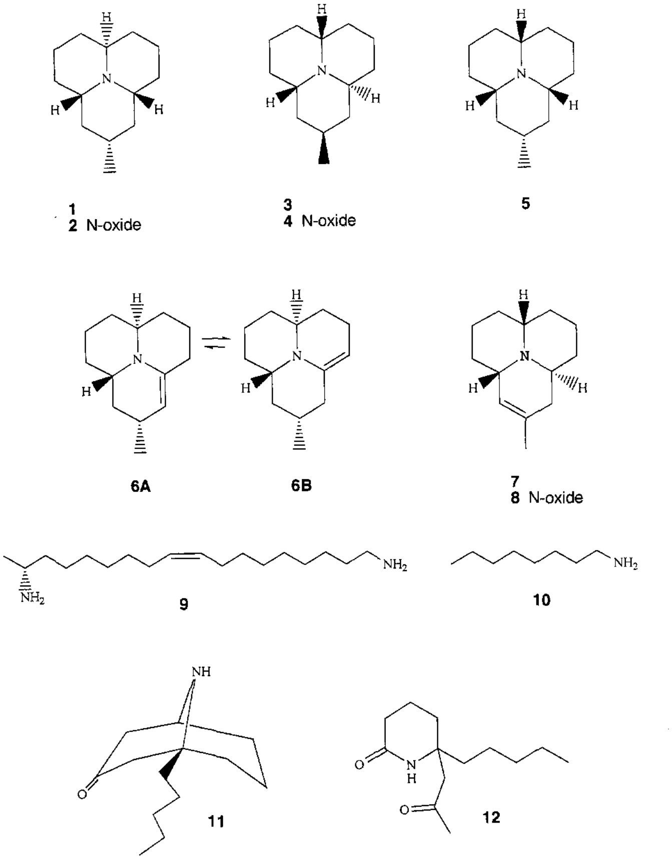


Fig. 2 Alkaloids found in the Coccinellini

vergine hydrochloride was very small in methanol even at 365 nm, which is not unexpected because the dissymmetry of the molecule is only due to the presence of a methyl group. Now, it could be predicted that the 2-methylperhydro-9b-azaphenalene ring system should possess only three ring junctions stereoisomers: one examplified by precoccinelline [1] and coccinelline [2] may be considered as the cis, trans, cis isomer (describing the relationship between the nitrogen lone pair and the ring junctions hydrogens); the second isomer (cis, cis, trans) is chiral and examplified by hippodamine [3] and convergine [4]; the third isomer (trans, trans, trans) has a plane of symmetry, like [1] and [2], but a different three-dimensional shape: it has a flat structure with the three rings in the same plane, whereas the two other isomers have two rings in one plane and the third one in a nearly perpendicular plane (Fig. 3). The third stereoisomer was found to be also naturally-occurring when myrrhine [5] was isolated from another Coccinellini, *Myrrha 18-guttata* (Tursch *et al.* 1975). The structure of myrrhine was established on the basis of its spectral properties (much more intense Bohlmann bands than [1] and [3], and by chemical correlation with coccinelline. Myrrhine could be oxidized in the laboratory to its *N*-oxide, but the latter was much less chemically reactive than coccinelline and convergine, presumably because of the shielding of the N-O function by six 1,3-diaxial interactions with hydrogen atoms.

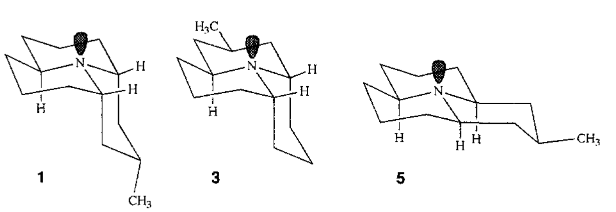


Fig. 3 Three-dimensional structures of precoccinelline [1], hippodamine [3] and myrrhine [5]

A dehydroderivative of precoccinelline, propyleine, was isolated from *Propylaea 14-punctata* and its structure proposed to be [6A] on the basis of its spectroscopic properties (Tursch *et al.* 1972). It was later established by synthesis that this compound exists as a rapidly interconverting mixture of [6A] (minor) and [6B] (major) (Mueller & Thompson 1980). Subsequently, hippodamine [3] and convergine [4] were found in another *Hippodamia* species, *H. caseyi* from Canada, together with their dehydroderivatives hippocasine [7] and hippocasine N-oxide [8]. The structure of the latter was also secured by an X-ray diffraction analysis (Ayer *et al.* 1976). Hippodamine [3], is also present in one species of the genus *Anisosticta*, *A. 19-punctata* (Tursch *et al.* 1975).

A recent observation of our laboratory concerning the 2-methylperhydro-9b-azaphenalene alkaloids should be mentioned here (unpubl.). We recently carried out a reinvestigation by capillary gas chromatography and GC/MS of some of the species that we studied 25 years ago. Surprisingly, we found that in *C. 7-punctata*, precoccinelline [1] is accompanied by a small amount of hippodamine [3] and coccinelline [2] by a small amount of convergine [4]. Conversely, in *H. convergens*, [3] and [4] are in a mixture with small quantities of [1] and [2], respectively. Thus it seems that the alkaloid content of these beetles is more complex than previously described. These results also indicate that the stereochemical control during the biosynthesis of these alkaloids is not as perfect as it was once thought to be. It should also be emphasized that the identification of the three different stereoisomers of the 2-methylperhydro-9b-azaphenalene is not a simple task. Indeed, [1] and [3] display the same tlc behaviour on alumina or silica gel. Moreover, [1], [3] and [5] have very close retention times on OV 1 and PEG capillary columns, and their mass spectra are nearly identical. The infra red spectra allows to distinguish [5] from [1] and [3], but the identification of the two latter compounds on this basis is rather difficult. Consequently, a clear-cut identification of these stereoisomers needs the use of high field ^{1}H and ^{13}C NMR and of reference compounds. Thus, it could be expected that some of the identifications previously reported in this series could be erroneous. Indeed, we have recently re-examined a sample of *Micraspis 16-punctata*, originally reported to contain precoccinelline [1]. We have now demonstrated by capillary GC and NMR that it contains in fact a mixture of [1] and hippodamine [3], the latter being the major compound (unpubl.).

A long chain diamine, harmonine or (17*R*) (*Z*)-1,17-diaminooctadec-9-ene [9] was isolated from four Coccinellini species: *Harmonia 4-punctata*, *H. leis conformis*, *Adonia variegata* and *Semiadalia 11-notata*. Its structure was determined by chemical and spectroscopic methods and its absolute configuration by a lanthanide-induced shift NMR study on the MTPA derivative (Braconnier *et al.* 1985a,b). Harmonine was also shown to be present, together with hippodamine [3], in a sample of *Hippodamia convergens*, surprisingly devoid of convergine [4], but which contained n-octylamine [10] (Braconnier *et al.* 1985a).

We will conclude this survey of the Coccinellini with two species of the genus *Adalia*, namely *A. 2-punctata* and *A. 10-punctata*. In these two species the major alkaloid was found to be the homotropane adaline [11], whose structure was again secured by an X-ray diffraction study on the hydrochloride (Tursch *et al.* 1973a), and its absolute configuration by circular dichroism (Tursch *et al.* 1973b). It was recently found that, in these two species, adaline is accompanied by a minor piperidine alkaloid, adalinine [12], the structure of which was established by two dimensional NMR experiments at 600 MHz (Lognay *et al.* 1996). An hypothetical relationship between these two compounds has been proposed (see Biosynthesis section).

To sum up, the tribe Coccinellini *sensu* Sasaji is by and large characterized by the production of alkaloids based on the 2-methylperhydro-9b-azaphenalene ring system. Within this general trend, some subtle tuning does seem to exist: coccinelline [2] and its free base [1] seem always to be the major alkaloids in the genus *Coccinella*, whereas convergine [4], its free base [3] and dehydroderivatives [7], [8] seem to dominate the chemistry of the genera *Hippodamia* and *Anisosticta*. On the other hand, the genus *Adalia* seems to depart from this general trend, as it is characterized by the production of the homotropane adaline [11]. However, the latter could be closely related from a biosynthetic point of view to the 2-methylperhydro-9b-azaphenalene alkaloids (see Biosynthesis section) (Tursch *et al.* 1973a). The chemotaxonomic significance, if any, of the presence of harmonine [9] in several species of Coccinellini is not clear at the present time.

In the subfamily Chilocorinae, only two species of one tribe, the Chilocorini have been chemically studied (Fig. 4). *Exochomus 4-pustulatus* was found to contain the complex hexacyclic alkaloid exochomine. Its structure and absolute configuration depicted in [13] were again secured by an X-ray diffraction analysis on its hydrochloride (Timmermans *et al.* 1992). This compound is a 'dimer' made up of the familiar 2-methylperhydro-9b-azaphenalene skeleton linked by a single bond to a 3,4-dimethyloctahydro-8b-azaacenaphtylene ring system, which probably has a biogenetic origin similar to that of the former, but a different cyclization pattern. The second Chilocorini species studied, *Chilocorus cacti* contained several alkaloids closely related to the former. The structure of the heptacyclic chilocorine A [14] was deduced from comparison of its spectral properties with those of exochomine [13] from which it differs by having two C-C linkages between the two tricyclic partners (McCormick *et al.* 1994). Very recently, the structure of the spirocyclic chilocorine B [15] was determined by an X-ray diffraction analysis which established its relative configuration (Shi *et al.* 1995). It is probably not by chance that the only three 'dimeric' alkaloids to have been found till now in the Coccinellidae originate from Chilocorini species, but it is too early to draw any chemotaxonomic conclusion from these data.

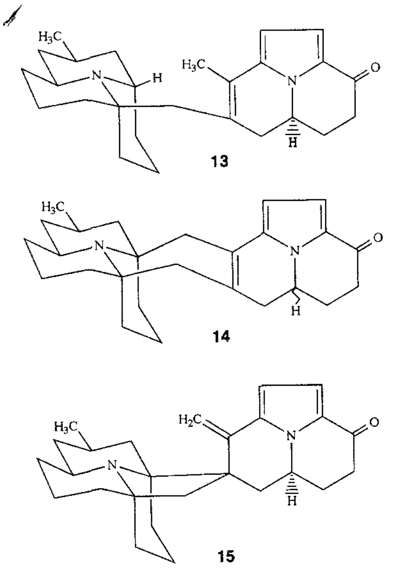


Fig. 4 Dimeric alkaloids from the Chilocorini

In the subfamily Epilachninae, there is only one tribe, the Epilachnini (Fig. 1). In contrast to the previously discussed coccinellid subfamilies it is predominantly phytophagous. Only three species of this group have been chemically studied, two in the genus *Epilachna* and one *Subcoccinella*. The Mexican bean beetle, *Epilachna varivestis*, is the most versatile alkaloid pro-

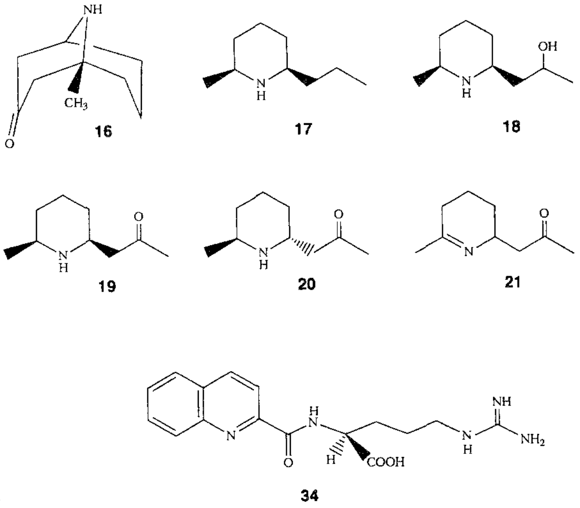


Fig. 5 Alkaloids from adult Epilachninae and (in part) from *Cryptolaemus montrouzieri* (Scymninae)

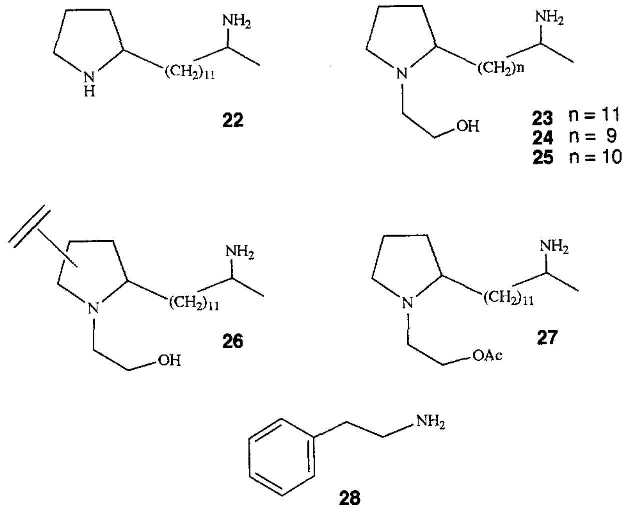


Fig. 6 Pyrrolidine alkaloids from *Epilachna*

ducer of all ladybird species so far examined. Indeed, not less than 17 alkaloids have been isolated from the different life stages of that beetle (Figs. 5–7). The first alkaloid to have been described from adult beetles was the homotropane euphococcinine [16] (Fig. 5) which is an homolog of adaline [11], bearing a methyl instead of a n-pentyl side chain (Eisner *et al.* 1986). In subsequent investigations, complex mixtures of simple piperidines [17]–[19], [21] (Fig. 5) and long chain pyrrolidines [22]–[27] (Fig. 6) were also identified in adult beetles, together with 2-phenylethylamine [28] (Attygalle *et al.* 1993a; Proksch *et al.* 1993). Compound [27] could well be an artifact of the isolation procedure (Proksch *et al.* 1993). Finally, a series of novel azamacrolides, epilachnene [29] and closely related derivatives [30]–[33] (Fig. 7), were reported from the pupal defensive secretion of that same beetle (Attygalle *et al.* 1993b). Pyrrolidine [23] was shown to be also present in the adults and larvae of *Epilachna guttattopustulata* from New Guinea (unpubl.). On the other hand, a new quinoline alkaloid, N_α-quinaldyl-L-arginine [34] (Fig. 5), was recently found in the European *Subcoccinella 24-punctata*. This compound was shown not to be from dietary origin (Wang *et al.* 1996).

In the subfamily Scymninae, only one species of the tribe Scymnini has been reported to contain alkaloids, that is the Australian ladybird *Cryptolaemus montrouzieri*. This species contains euphococcinine [16], also found in *E. varivestis*, together with the piperidine alkaloid [19] and its trans isomer [20] (Fig. 5) (Brown & Moore 1982). This result could point to some relationship between the Scymnini and the Epilachnini, but the number of species studied is too small to draw any conclusion.

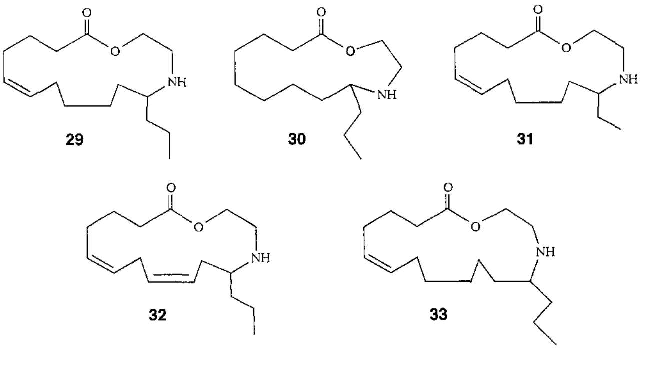
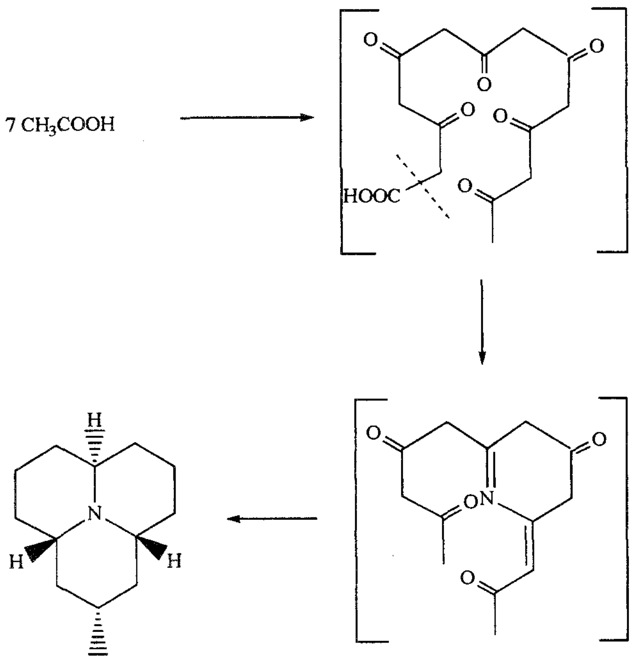


Fig. 7 Azamacrolides from the pupal hairs of *E. varivestis*

To conclude this section, it should be mentioned that some typical ladybird alkaloids have also been isolated from other animal sources. An unidentified 2-methylperhydro-9b-azaphenalene was reported to be a volatile constituent of male and female boll weevils (*Anthonomus grandis*) (Hedin *et al.* 1974), whereas precoccinelline [1], hippodamine [3] and propyleine [6A,B] were found in the defensive secretion of the prothoracic glands of the soldier beetle *Chauliognathus pulchellus* (Cantharidae). Due to the phylogenetic distance between Coccinellidae and Cantharidae, the occurrence of the same compounds in the two groups of beetles was considered to be a case of convergent evolution (Moore & Brown 1978). Most unexpectedly, precoccinelline [1] was also isolated as a minor component from the skin of bufonid toads (Garraffo *et al.* 1992) and dendrobatid frogs (Daly *et al.* 1994). However, it was found that frogs raised in captivity on an artificial diet are totally lacking alkaloids. Thus it seems likely that precoccinelline has its origin in the leaf-litter prey of the frogs (Daly *et al.* 1994).

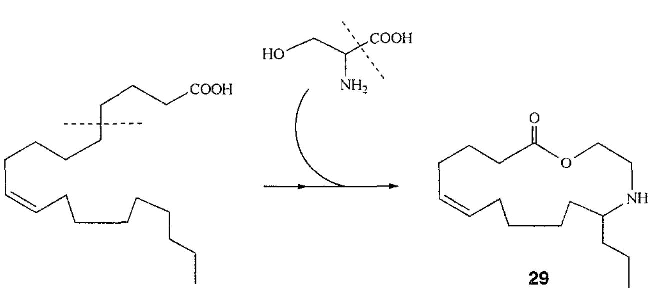
Biosynthesis

Right from the beginning of the studies on ladybird alkaloids, it has been suspected that these compounds are produced *de novo* by the beetles, because neither coccinelline [2] nor precoccinelline [1] could be detected in the aphids that constitute the prey of *C. 7-punctata* (Tursch *et al.* 1971b). This is also true for phytophagous species, for example *E. varivestis* (Proksch *et al.* 1993) and *Subcoccinella 24-punctata* (Wang *et al.* 1996). However, the autogenous production of alkaloids by ladybirds has been unambiguously proved only in three cases. Specimens of *C. 7-punctata* were fed with [1-^{14}C] and [2-^{14}C]acetate, and the labelled samples of coccinelline were submitted to a Kuhn-Roth degradation, which afforded acetic acid, corresponding to carbon atoms 2 and 10 of the alkaloid. In the two experiments, acetic acid was isolated as its 2-aminonaphtalene derivative which was shown to contain about 16% of the original label of coccinelline. These results are in agreement with a polyketide origin of these alkaloids. They also allowed us to exclude a biogenetic scheme involving the condensation of six acetate units followed by methyl addition, since the relative specific activities in the two labelling experiments would then be 16.7 and 0% respectively (Tursch *et al.* 1975). The precise pathway leading to the 2-methylperhydro-9b-azaphenalene skeleton is not known but could be as depicted in scheme 1.



1
2 N-oxide
35

Scheme 1 Hypothetical biogenetic scheme for precoccinelline [**1**] and coccinelline [**2**]



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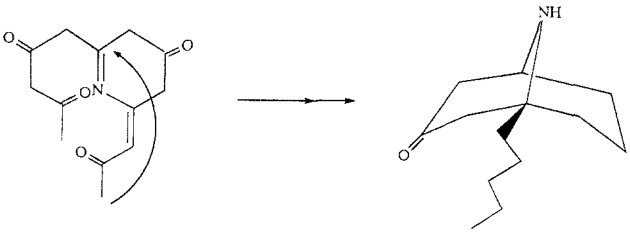
Scheme 3 Biogenetic scheme proposed for epilachnene [**29**]

We have recently undertaken experiments to delineate the biosynthetic scheme of the homotropane adaline [11]. By feeding [1-^{14}C]acetate to specimens of *A. 2-punctata*, we isolated radioactive adaline, but no degradation of the radioactive sample has yet been performed (unpubl.). However, these results indicate that adaline could also be a polyacetate, and thus bring some support to the hypothetical biogenetic relationship between adaline and the 2-methylperhydro-9b-azaphenalene alkaloids put forward by Tursch *et al.* (1973a). Thus, compound [35] of scheme 1, which is a plausible intermediate on the way to coccinelline and derivatives, could lead to adaline [11] by an alternative cyclization which is depicted in scheme 2. The only other biosynthetic work of which we are aware concerns the azamacrocycle epilachnene [29], from the glandular hairs of the pupa of the Mexican bean beetle, *E. varivestis*. Experiments with ^{2}H-labelled oleic acid, ^{2}H-labelled L-serine and ^{13}C,^{15}N-labelled L-serine established the origin of the alkaloid's entire skeleton, which is formed as shown in scheme 3, with loss of four carbon atoms from the carboxyl end of oleic acid (Attygalle *et al.* 1994). Thus, the results available till now point to a polyketide/fatty acid origin for some, if not all, ladybird alkaloids.



35
11

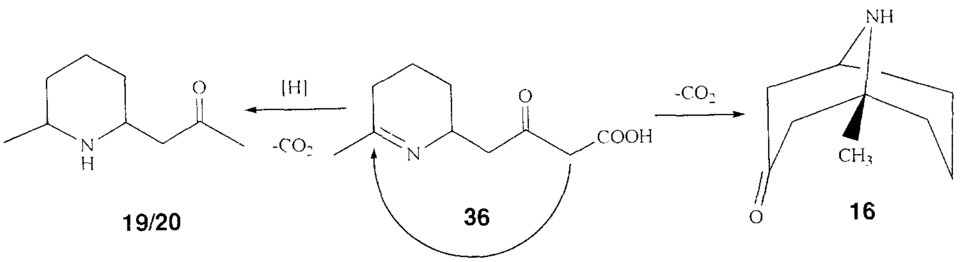
Scheme 2 Hypothetical relationship between coccinelline [**2**] and adaline [**11**]

Besides these experimental works, suggestions can be made as to the possible interrelationship between some of these alkaloids. For example, a piperidine derivative such as [36], closely related to the piperideine [21], found in *E. varivestis*, could be the common precursor of piperidines [19] and [20], and of the homotropane euphococcinine [16] (Scheme 4). On the other hand, a biosynthetic relationship between adaline [11] and the piperidine alkaloid adalinine [12], both coexisting in *Adalia 2-punctata* has been proposed (Scheme 5) (Lognay *et al.* 1996). The possible biogenetic relationships between the 'juvenile' [22]–[27] and 'imago' [16], [18], [19] alkaloids of *E. varivestis* have also been discussed (Proksch *et al.* 1993).

Mode of release

The phenomenon of reflex bleeding in adult Coccinellidae has been repeatedly studied in the past. The reflex fluid was only observed to exude from the joint between the femur and tibia, and the beetles were able to reflex bleed from all six legs. Also, it was possible to induce each leg to produce reflex fluid independently (Cuénot 1896; Hollande 1911, 1926; Happ & Eisner 1961). The conclusions of these authors have recently been confirmed by Holloway *et al.* (1991). They also showed that, in *C. 7-punctata*, a seven day interval was only sufficient to enable the beetles to reflex bleed from between 60 and 70% of full capacity. In contrast, the rate at which *A. 2-punctata* replenished their reserves of fluid was considerably faster (de Jong *et al.* 1991).

Some controversy exists in the literature as to the precise nature of the emitted fluid. Most of the authors (Cuénot 1896; Hollande 1911, 1926; Happ & Eisner 1961) came to the conclusion that it is identical with haemolymph. However, Kay *et al.* (1969) reported that they found no blood cells in the fluid of *C. 7-punctata*. In a recent study of the reflex bleeding of *Exochomus 4-pustulatus*, Pasteels & Cingolani (unpubl.) confirmed the prevalent view that the emitted fluid is indeed



Scheme 4 Hypothetical biogenetic relationship between piperidine and homotropane alkaloids in *E. varivestis* and *C. montrouzieri*

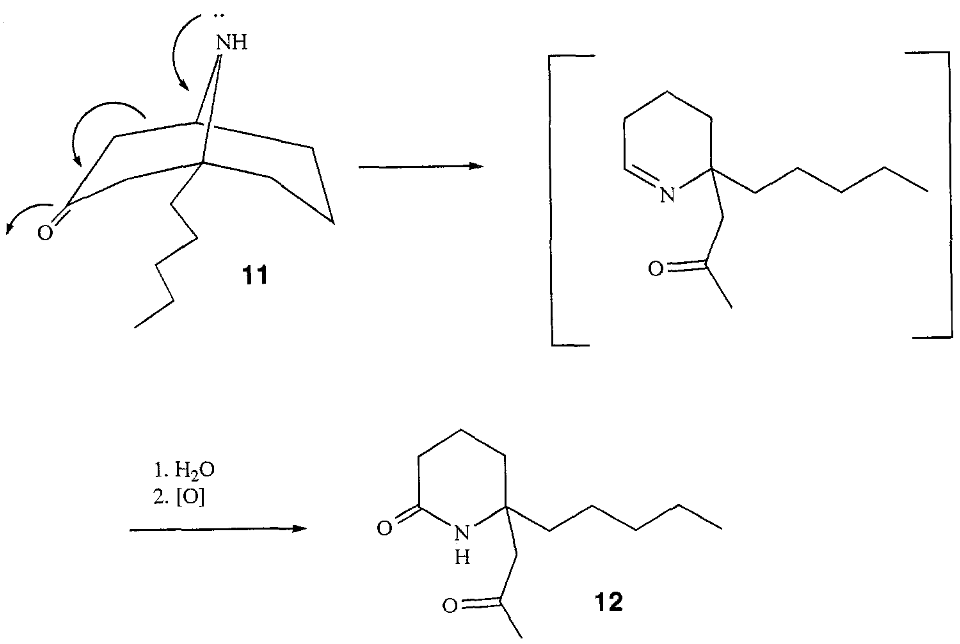
blood. The reasons for these conflicting observations are not clear at the time being. More important, probably, is the finding that in the three species mentioned above the reflex fluid does contain the alkaloid(s) characteristic of that species. This is also true for *E. varivestis*, which exudes euphococcinine [16] (Eisner *et al.* 1986). In some species, the alkaloid concentrations have been measured, but this topic will be discussed in the last section.

Reflex bleeding has also been described for the larvae of some species, such as *C. 7-punctata*, *2-punctata* and *Exochomus 4-pustulatus* (Hollande 1911; Kendall 1971). In the latter insect, reflex bleeding takes place in all larval stages when the membrane of specialized areas of the tegument, called pores, are breaking off (Kendall 1971). The breaking off is triggered by the contraction of muscles, which allow the larva to reflex bleed from particular points of the tegument, notably those that are close to the stimulation (Pasteels & Cingolani unpubl.).

In some cases, the pupae also have been shown to exhibit reflex bleeding, e.g. *E. auritus* (Hollande 1911) and *E. 4-pustulatus*. In the latter case, the mechanism is similar to that of the larvae. In two other species, *A. 2-punctata* and *Rhizobius forestieri*, the pupae seem to be devoid of reflex bleeding (Pasteels & Cingolani unpubl.). Finally, in *E. varivestis*, the pupae are protected by specialized hairs which release droplets containing the azamacrolide alkaloids [29]–[33] (Attygalle *et al.* 1993b).

Alkaloid variation throuth the life cycle

From an ecological point of view, it would be important to know if vulnerable stages of the life cycle like the egg and pupa are also protected by defensive alkaloids. Unfortunately, few species of ladybirds have been studied for their alkaloid content through all the life stages. In *C. 7-puncata* and *A. 2-punctata* the adult alkaloids have also been found in the eggs and larvae (Pasteels *et al.* 1973; Lognay *et al.* 1996). This same situation pertains for *E. 4-pustulatus*, the pupa of which contains also exochomine [13] (Pasteels & Cingolani unpubl.). These results would suggest that in ladybirds, the chemical protection of all life stages is due to the same alkaloid(s), which are probably transmitted by the female to the egg. That this conclusion is not generally valid was demonstrated by two independent studies of the alkaloid content of *E. varivestis* (Attygalle *et al.*



Scheme 5 Possible relationship between adaline [**11**] and adalinine [**12**] in *Adalia* species

Table 2 Repellency of ladybird alkaloids on ants (concentration in M/l)[a]

Alkaloid	Ant species	RD_{50}[b]	Threshold	[c] in haemolymph	Reference
2	*Myrmica rubra*	$1 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	$3 \cdot 10^{-2}$	Pasteels *et al.* 1973
4	*M. rubra*	$7 \cdot 10^{-4}$	$2 \cdot 10^{-4}$	–	Pasteels *et al.* 1973
3	*M. rubra*	–	$1 \cdot 10^{-4}$	–	Braconnier *et al.* 1985a
16	*Monomorium pharaonis*	$1 \cdot 10^{-3}$	[c]	$1 \cdot 10^{-2}$	Eisner *et al.* 1986
13	*Lasius niger*	–	$1 \cdot 10^{-4}$	$1 \cdot 10^{-1}$	Pasteels & Cingolani unpubl.
34	*Myrmica rubra*	$1 \cdot 10^{-4}$	–	$1 \cdot 10^{-2}$	Wang *et al.* 1996

[a] For testing procedure see text
[b] Concentration which repels 50% of ants
[c] Not active at 10^{-4} M

1993a; Proksch *et al.* 1993). It was found that eggs, larvae and pupae accumulate only pyrrolidine alkaloids [22]–[27] (as well as azamacrocyles [29]–[33] in pupal hairs). Imagines, for the first two days after adult emergence, resembled pupae with regard to the alkaloids present. In older (10–14 d) imagines however, the pyrrolidines were progressively replaced by piperidines [17]–[19] and [21] and by homotropane [16], which are characteristic of the adult stage. Whether these 'adult' alkaloids are synthesized independently or originate from the 'juvenile' pyrrolidines is not clear at the time being (Proksch *et al.* 1993). Moreover, the biological significance of these ontogenic variations, as opposed to the apparent constancy in the other species studied till now, remains obscure.

Repellent and toxic activities of the alkaloids: efficiency of the protection

Whatever its efficiency and sophistication, there is no defence mechanism which renders its possessor immune to the attack of any predator. This is illustrated by the relationship between ladybirds and predators, particularly birds, which is discussed at length by Majerus (1994), and consequently will only be alluded to here. For example, whereas *C. 7-punctata* specimens are unpalable for captive quails (*Coturnix coturnix*) (Pasteels *et al.* 1973), and lethal to blue tits (*Parus caeruleus*) nestlings (Marples *et al.* 1989), house martins (*Delichon urbica*) do feed on ladybirds without being harmed (Majerus 1994). In another study, coccinellids were found to be unacceptable to various predators, including mammals, birds, lizards and one toad (Frazer & Rothschild 1960). One-day-old *E. varivestis* beetles were promptly taken and eaten by jumping spiders (*Phidippus regius*), whereas 7-day-old beetles were either quickly rejected uninjured or killed but only partially eaten. This behaviour is to be correlated with the increase of euphococcinine [16] concentration with age in this species (Eisner *et al.* 1986).

The repellent effect of ladybird alkaloids was also tested by several authors, in a bioassay that allows thirsty ants to choose between a 10^{-1} M aqueous sucrose solution (control) and 10^{-1} M aqueous sucrose with added alkaloid. The results summarized in Table 2 show that the six alkaloids tested so far are all repellent at 10^{-3} M, with the most active [9], [13], [34] being still repellent at 10^{-4} M. In cases where the alkaloid concentration in the haemolymph or reflex blood has been estimated, it was always equal to or higher than the concentration at which it is repellent (Table 2). These tests show that these alkaloids are able to efficiently protect ladybirds against arthropod predators. The same tests were performed on different species of ladybirds with exochomine [13], the alkaloid of *E. 4-pustulatus* (Pasteels & Cingolani unpubl.). Interestingly, it was found that at 10^{-2} M, exochomine is not repellent to *E. 4-pustulatus* itself, nor to *Rhizobius forestieri*, but well to *Cryptolaemus montrouzieri*. Thus it seems that, at least in this particular case, the protection afforded by exochomine is not sufficient to prevent either cannibalism or predation by some other coccinellid species particularly on eggs and larvae.

The amounts of coccinelline [2] in the reflex fluid and in different parts of body of *C. 7-punctata* after they were reflex bled was determined by Holloway *et al.* (1991). They found that the concentration of [2] in the reflex fluid (6–11 µg/mg of fluid) was about 10 times higher than in the other parts of the body. Moreover, the amount of fluid produced built up rapidly following winter hibernation and, as the increase in coccinelline production was not as great, the concentration of the alkaloid declined slowly to a steady value (about 4 µg/mg of fluid). Significant variations were also found among beetles in the amount of fluid produced (for males and for females corrected for body weight) and the coccinelline concentration of the reflex blood. Similar results were obtained for *A. 2-punctata*, although the adaline concentration in the reflex blood was much higher (53 µg/mg), maybe in order to compensate for the lower repellency and toxicity of adaline (Marples *et al.* 1989) in comparison with coccinelline (de Jong *et al.* 1991).

The conclusion of Holloway *et al.* (1991) that the concentration of coccinelline in the reflex fluid is much higher than in other parts of the body may be criticized because the authors did not measure the concentration of the alkaloid in the haemolymph before reflex bleeding. Indeed, in the first quantitative study of alkaloid production by all life stages of a ladybird, Pasteels & Cingolani (unpubl.), working on *E. 4-pustulatus*, did not find such a difference. The amounts and concentration of exochomine in the different life stages were

Table 3 Amounts (in μg and % of fresh weight) of [13] in different life stages of *Exochomus 4-pustulatus* (from Pasteels & Cingolani unpubl.)

Stage	Amount	% of fresh weight
1 day old eggs	0.8	–
5 day old eggs	0.85	–
1st instar	0.6	0.32
2nd instar	2.5	0.17
3rd instar	15.9	0.28
4th instar	29.9	0.17
pupa	38.4	0.25
female	40.0	0.30
male	25.9	0.26

determined by HPLC and are reported in Tables 3 and 4. Several interesting conclusions can be drawn from these values. Exochomine is present at all stages of the development. Its concentration in 1-day-old and 5-day-old eggs is the same, showing that there is no biosynthesis during embryogenesis. Moreover, the total amount of alkaloid increases with development, but the relative proportion is nearly constant, at least from the second instar larva, and corresponds to about 0.25% of the fresh weight. One of the most striking results of this study is the finding, in contrast with Holloway *et al.* (1991), that the alkaloid concentration was about the same in the haemolymph ($1 \cdot 10^{-1}$ M) and in the reflex fluid ($8 \cdot 10^{-2}$ M), once again indicating that the two are probably identical. It was also found that more than 30% of the adult alkaloid may be exuded during reflex bleeding.

Conclusions

The results presented here amply demonstrate that ladybirds are the most versatile and prolific alkaloid producers of the insect world. However, at the same time, this review shows that our knowledge of the defensive mechanisms of ladybirds is still severely limited.

On the plus side, 34 nitrogen-containing compounds have so far been isolated and their structure determined. Many of these compounds are new, and belong to structural classes that have no counterpart in plants, which explains why there has been such an endeavour towards their synthesis. In some cases, their toxic and repellent properties have been evaluated, and convincing evidence has been brought as to their protecting action against various types of predators.

Table 4 Concentration of [13] (in M/l) in haemolymph and reflex fluid in different life stages of *Exochomus 4-pustulatus* (from Pasteels & Cingolani unpubl.)

Stage	haemolymph	reflex fluid
4th instar	$2.8 \cdot 10^{-2}$	$3.7 \cdot 10^{-2}$
pupa	$3.0 \cdot 10^{-2}$	$4.7 \cdot 10^{-2}$
female	$1.1 \cdot 10^{-1}$	$8.3 \cdot 10^{-2}$
male	–	$7.7 \cdot 10^{-2}$

However, it cannot be too much emphasized that this field of research is still in its infancy. Indeed, only a very small part of the existing species have been investigated and most of them belong to one tribe, the Coccinellini (Table 1). Thus, few chemotaxonomic generalizations can yet be made, and nothing can be said about the frequency of alkaloid production in this family of insects. Thus, other subfamilies, especially those which have been neglected till now, should be examined. Moreover, very few data exist concerning the sequestration of defensive compounds from the prey or from the host plant. Finally, only two biosynthetic studies devoted to these beetles have so far been published. In both cases the beetles were found to biosynthesize their alkaloids *de novo*, but more studies are needed to assess the generality of this finding.

To sum up, much more chemoecological work must be done before the questions raised above can be answered and before the biological and pharmacological activities of ladybird alkaloids and their ecological relevance for ladybird adaptation and survival could be properly evaluated.

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