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Chemical defense of the ladybird beetle *Epilachna paenulata*

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Summary. The defensive chemistry of the ladybird beetle *Epilachna paenulata* (Coleoptera: Coccinellidae) was characterized as a mixture of piperidine, homotropane and pyrrolidine alkaloids. Whole body extracts of adult beetles contain four major alkaloids: 1-(6-Methyl-2,3,4,5-tetrahydro-pyridin-2-yl)-propan-2-one; 1-(6-methyl-2-piperidyl)-propan-2-one; 9-aza-1-methyl-bicyclo[3.3.1]nonan-3-one and 1- (2´´- hydroxyethyl)-2-(12´-aminotridecyl)-pyrrolidine. Comparative studies of the defensive chemistry of eggs, larvae, pupae and adults showed both qualitative and quantitative differences in alkaloid composition among the four life stages, and also within adult age. Laboratory predation bioassays with wolf spiders showed that the adults are better protected than the larvae and pupae. Field tests showed the adult alkaloid extract to be deterrent to ants.

Key words. *Epilachna paenulata* – Coleoptera – Coccinellidae – alkaloids – chemical defenses – ontogenetic variation

Introduction

Coccinellid beetles contain a variety of defensive alkaloids that render them unpalatable to several predators. These alkaloids are believed to be mostly of endogenous origin, and include perhydroazaphenalenes, azabicyclononanes (homotropane), aliphatic and aromatic amines, pyrrolidine and piperidine alkaloids, azamacrolides and macrocyclic polyamines. Such chemical load, displayed by reflex bleeding in several species, may correlate to the bright coloration and distinctive odour shown by most species in the family (Daloze *et al*. 1995; King & Meinwald 1996).

Among coccinellids, the genus *Epilachna* (Coleoptera: Coccinellidae) has been a source of several defensive alkaloids that occur systemically in all life stages. Two species from Central and North America (Gordon 1985; Tissot 1943), *Epilachna varivestis* Mulsant 1846 and *Epilachna borealis* Fabricius 1775, have been extensively studied. *E. varivestis* adults contain a complex mixture of alkaloids, including piperidine (i.e. **1**), homotropane (i.e. **3**), and pyrrolidine alkaloids (i.e. **4** and its analogue with no hydroxyethyl moiety) (Attygalle *et al.* 1993a; Eisner *et al.* 1986; Proksch *et al.* 1993). All immature stages as well as newly emerged adults accumulate only pyrrolidine alkaloids (**4** and other related structures) (Proksch *et al.* 1993). Interestingly, older adults (11-days) contain mainly the homotropane **3**. In addition, oily droplets from glandular hairs of pupae contain the azamacrolide epilachnene, a macrocyclic lactone with anti-insectan activity (Attygalle *et al*. 1993b). In the case of *E. borealis*, Radford *et al*. (1997) reported that alkaloid extracts from young adults showed a similar GC profile than *E. varivestis* adult extracts, with larger amounts of a novel biciclyc alkaloid [5-(12'-amino-tridecyl)pyrrolidinoöxazolidine] also found in *E. varivestis*. *E. borealis* chemical repertoire proved to be surprisingly more complex, since the defensive secretion from pupal hairs contains a mixture of macrocyclic polyamines (Schroeder *et al*. 1998a,b).

Epilachna paenulata Germar 1824 (Coleoptera: Coccinellidae) is a South American ladybird beetle that feeds on plants of the family Cucurbitaceae (melon, water-melon, squash). These beetles are most active during summer, when they can cause serious damage to plants grown under "organic conditions" (Scatoni & Bentancourt 1999). The possibility of using native predators as biological control agents, and the chemical versatility shown by other members of the genus (Attygalle *et al.* 1993a,b; Eisner *et al.* 1986; Proksch *et al.* 1993; Schroeder *et al*. 1998a,b), prompted us to undertake the study of *E. paenulata* defensive chemistry. Here we present our results, with respect to chemical composition, bioactivity and ontogenetic variation.

Materials and Methods

Epilachna paenulata

A laboratory colony was maintained on squash (*Cucurbita pepo*) under controlled conditions of temperature (20 °C ± 2) and light (14:8). For the initial settlement of the colony, individuals were collected on squash plants at organic farms nearby Montevideo, and new field-collected individuals have been added every year. Individual life stages were maintained separately confined in cages (30 × 30 × 30 cm), in which 9-12 plants (3 weeks old) were added every 3-4 days.

Bioassays with individuals

E. paenulata were tested in its defensive capacity against a generalist predator (wolf spiders of the genus *Lycosa*). Ten pupae (2 days old), larvae (4th instar) and adult beetles (4-5 days old) were individually offered in closed, sand-filled containers (10 cm diameter, 12 cm height) to different spiders that had been starved for ten days prior to the tests. The results were scored as rejection only if the spiders accepted a larva of *Tenebrio molitor*, an edible item, after attacking the beetle.

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The pupae were also tested in the field against the generalistic predatory ant *Linepithema humile* (Hymenoptera: Formicidae). The pupae (N = 7) were individually placed close (2 cm) to different nest entrances of *L. humile*. The number of ants contacting the pupae was observed every 15 minutes for 4 hours, and every hour afterwards, up to a total of 10 hours for the entire bioassay.

Bioassays with alkaloid mixture

The deterrence of the alkaloid extract from adult beetles was evaluated using a choice experiment in the field. Two freshly-dead larvae of *T. molitor* were placed on the ground, two to three centimeters apart, in twelve sites within a grassy area of 500 m^2. One larva was treated topically with 12 µl of a dichloromethane solution of an alkaloid extract containing 0.6 µg/µL of alkaloids, so that a dose of 7 µg – representative of the alkaloid content of one *E. paenulata* adult - was applied to the mealworm. The control larva was treated with 12 µl of dichloromethane. The larvae were checked every 10 minutes, recording the number and kind of organisms that encountered the mealworms, and the fate of the mealworm larvae.

Statistical analyses

Frequencies of accepted and rejected individuals (laboratory assays with spiders) were analyzed with chi-square goodness of fit test (Conover 1980). Data from the ant bioassay with the alkaloid mixture were subjected to a repeated measures analysis of variance (time as repeated measure, Conover 1980).

Alkaloid extraction and identification

Individuals of different developmental stages were obtained from our laboratory colony, frozen at -20 ºC, and extracted with MeOH at room temperature (3 h). The extracts were dried under vacuum, re-suspended in 0.2 N HCl and washed with Et_2O. The solution was further acidified with HCl and left overnight with Zn dust. The Zn was filtered and the solution was taken to pH 10 with NaOH. The alkaloids were then extracted with CH_2Cl_2, obtaining thereby a crude extract from eggs, larvae, pupae and adults. To isolate and identify the alkaloids, the crude adult extract was further purified by column chromatography using neutral Alumina and CH_2Cl_2:MeOH mixtures (0 to 20% MeOH). The pure alkaloids were then analyzed by NMR (^1H, ^{13}C, COSY, HMQC, HMBC), and GC/MS. Alkaloids in the other life stages were identified by comparison of their respective GC/MS analyses.

Extractions were conducted with 240 adults (12 g fresh weight, final extract 42 mg), 36 pupae (1.0 g, final extract 5.6 mg), 34 larvae (1.6 g, final extract 4.5 mg), and an egg mass of 538 mg (final extract 7.4 mg).

Alkaloid quantification in individual beetles

The alkaloid contents of individual beetles in the different life stages were determined by GC analyses of extracts of larvae (3rd instar), pupae (3-4 days old), adults (0, 5 and 10 days old) and egg clusters laid by individual females. Extractions (N = 10 for each instar, N = 3 clusters for eggs) were performed under stirring (24 h) and at room temperature, with 1 mL of a solution of nicotine (25 µg/mL in MeOH:HCL 2N, 98:2), which served as internal standard ($r^2 = 0.996$, linear range 1.5 µg – 24 µg). Centrifuged extracts were then purified on individual ionic exchange SPE columns (Sulfonic, Baker) in three steps [i.1 mL MeOH:HCl 2N (98:2); ii.1 mL MeOH; iii.2 mL MeOH:NH_4OH conc. (91:9)]. Alkaloids were directly analyzed (GC) in fraction iii.

NMR and gas chromatography

NMR spectra were recorded using a Bruker DPX-400 Avance Spectrometer (400MHz for ^1H, 100 MHz for ^{13}C). GC/MS analyses were performed on a Shimadzu QP 5050 [Carbowax 20M, 25m, 0.32 mm; splitless injection in CH_2Cl_2), 60 °C (6 min)-240 °C (20 min) at 10 °C/min. Injector and interphase temperature: 250 °C]. GC analyses of individual extracts for alkaloid quantification were performed on a GC HP 5890 using the same chromatographic conditions [Injector and FID temperature: 250 °C]. Peak assignments were confirmed by GC/MS.

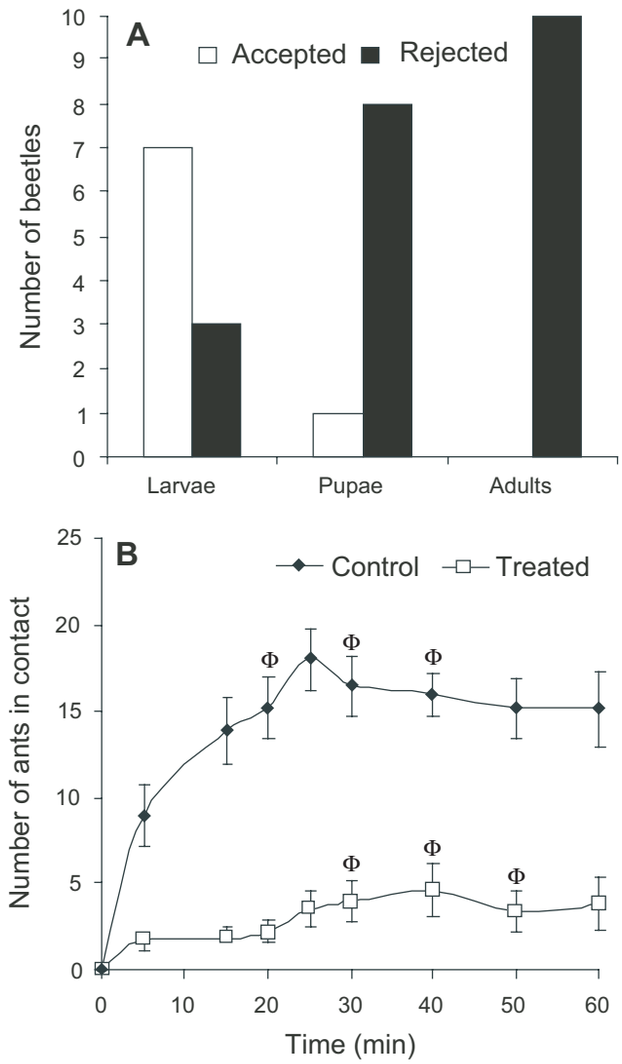

Fig 1 (A) Laboratory predation bioassays with different life instars of *E. paenulata* (all stages N = 10), against *Lycosa* spiders Differences were significant for pupae (χ^2 tests, $p < 0.05$. Larvae, $p = 0.2$). (B) Field feeding preference tests with *L. humile* ants. The symbol Φ over the trace indicates that a mealworm larva (either control or treated) was removed by the ants. Overall results are signicantly different ($p < 0.05$, Repeated measures test, Conover 1980)

Results

Bioassays with individuals

E. paenulata adults are well protected against wolf spiders (*Lycosa* spp.). All 10 adult beetles offered to the spiders were attacked immediately and rejected within seconds (Figure 1A). In all cases there was contact between the spider's palpi and the beetle; but none of the beetles were killed as a result of the

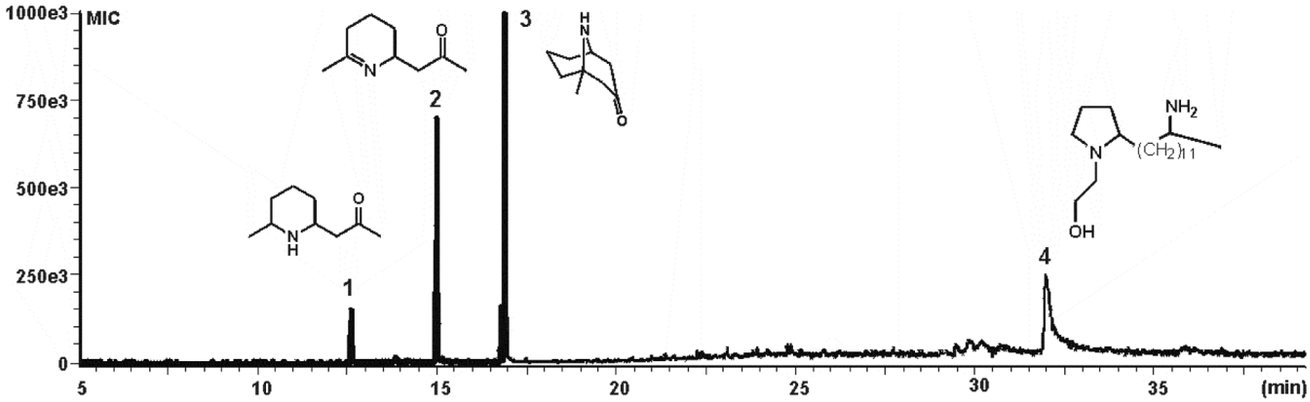

Fig 2 Gas chromatogram showing the four main alkaloids of *E. paenulata* adult extracts. For clarity, a Multiple Ion Chromatogram (MIC, m/z = 98, 110, 114) is shown

Table 1 Alkaloid content in different life instars of *E. paenulata*. Results are shown as µg of nicotine equivalent (mean ± SD; larvae, pupae and adults, N = 10 for; eggs, N = 3 clusters).

Life instar	Total individual alkaloid load (µg)	Alkaloid concentration (ng/mg wet weight)
Eggs	0.1 ± 0.1	50 ± 30
Larvae	1.0 ± 0.1	32 ± 2
Pupae	0.8 ± 0.1	16 ± 1
Adults		
0 days old	1.2 ± 0.3	35 ± 7
5 days old	4.6 ± 0.5	88 ± 9
10 days old	3.6 ± 0.3	71 ± 5

attack. Only 3 beetles showed reflex bleeding upon the attack, indicating that this reaction is not essential to elicit the rejection of the spider. After the attack, 5 spiders engaged in intensive cleansing of the mouthparts against the sandy substrate.

In the case of pupae, 9 out of 10 individuals were rejected by the spiders (10 min., χ^2=6.4, $p < 0.05$). However, no adults emerged from the 9 rejected pupae, indicating that significant injury resulted from the spider's attack. *E. paenulata* larvae were even more vulnerable to the spiders: out of 10 larvae tested, only 3 were rejected and one of these died with 24 hours (χ^2=1.6, p = 0.2).

The field bioassay with the pupae showed that these are not an acceptable prey item for *L. humile* ants. None of the seven *E. paenulata* pupae that had been placed next to the nest entrances were either taken away or injured by the ants. After the experiment, the pupae were kept in the laboratory and all emerged as adults shortly thereafter. During the 10-hour observation period, *L. humile* workers were very active around the pupae, including several individual contacts. However, no recruitment of nestmates was evidenced, in contrast with the behaviour observed when edible *T. molitor* were offered (see below).

Bioassays with secretion

The twelve sites settled in the field with the alkaloid-treated and control mealworm larvae were exclusively visited by two species of ants. *Linepithema humile* (Hymenoptera: Formicidae) arrived at 10 sites, and *Camponotus mus* (Hymenoptera: Formicidae) visited 2 sites. The results presented here correspond to the 10 sites visited by *L. humile*. Figure 1B shows the number of ants in contact with each of the mealworms, throughout the first hour after the first ant contact was observed. In all cases, *L. humile* workers showed a clear preference for the control larvae over the mealworm coated with alkaloid ($p < 0.001$, repeated measures analysis of variance). The number of ants in contact with the control larvae increased considerably within the first 30 minutes after the initial contact, suggesting some form of recruitment by the ants. In four sites, the ants took one or both mealworms to the nest (Figure 1B).

Alkaloid identification

Crude alkaloid extracts of adult beetles showed four major peaks (Figure 2). Two of the alkaloids (**3** and **4**) were identified on the basis of NMR and mass spectral data, as 9-aza-1-methyl-bicyclo[3.3.1]nonan-3-one (euphococcinine, **3**) and 1-(2´´-hydroxyethyl)-2-(12´-aminotridecyl)-pyrrolidine (**4**), both known within the genus *Epilachna* (Eisner *et al.* 1986; Proksch *et al.* 1993). **1** and **2** were identified by comparison of their mass spectra with data reported for two alkaloids from *E. varivestis* (Proksch *et al.* 1993), as 1-(6-methyl-2-piperidyl)-propan-2-one and 1-(6-methyl-2,3,4,

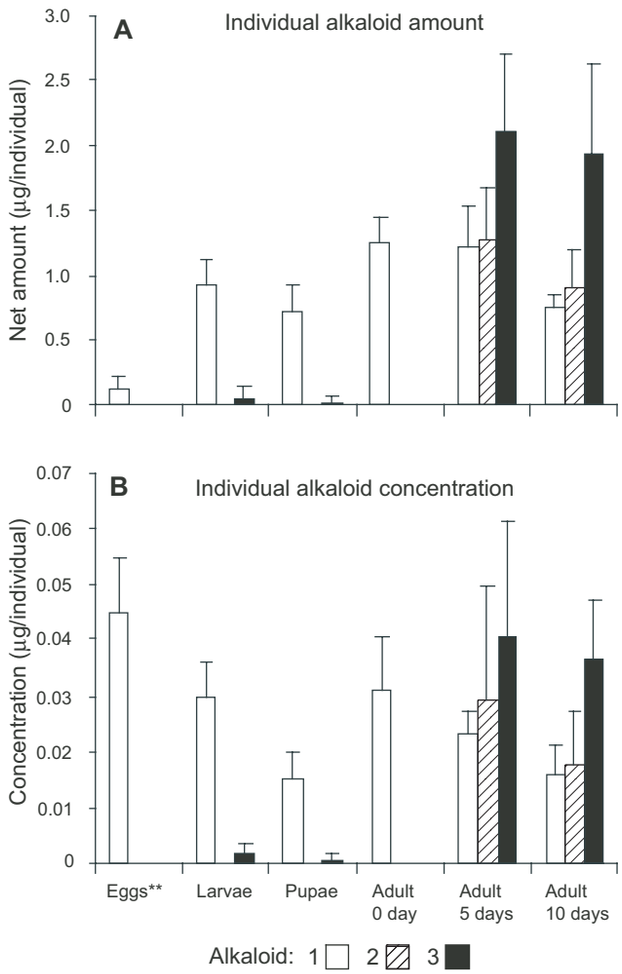

Fig 3 Individual alkaloid amount and concentration (in nicotine equivalents) in different life instars of *E. paenulata* (**, egg mass was estimated from data from a previous experiment). Numbers refer to alkaloids shown in Figure 2

5-tetrahydro-pyridin-2-yl)-propan-2-one, respectively. To confirm the imine double bound in **2,** the alkaloid extract from an individual adult was reduced with $NaBH_3CN$ in MeOH. Resulting GC analysis of the product showed the disappearance of **2** and the appearance of **1**, which had not been detected in this individual sample before the reduction. The other developmental stages contain only alkaloids **1** and **3**, but in different amounts (Figure 3).

Alkaloid quantification in individual beetles

The total alkaloid contents of the different life stages of *E. paenulata* are shown in Table 1. All immature stages as well as newly-emerged adults contained mainly the piperidine alkaloid **1** (Figure 3); no other alkaloid was detected in eggs or in newly emerged adults. Pupae and larvae also contain small amounts of the homotropane **3**, which was the main alkaloid in older adults (5 and 10 days old). These also contain the unsaturated piperidine **2**, in comparable amounts to the piperidine **1**. The pyrrolidine **4** was present only in older adult extracts in non-quantifiable amounts.

Discussion

Epilachna paenulata adults possess great defensive capacity against a generalist predator, as shown in the laboratory tests against *Lycosa* spiders (Figure 1A). Although many coccinellids show reflex bleeding as a defensive strategy, this behaviour does not seem to be essential for defense, suggesting that either the systemic alkaloids diffuse through the cuticle, or some odour or epicuticular component act as a warning device. Both scenarios are possible: the alkaloids themselves, as a purified extract, proved to be deterrent against ants when tested in the field (Figure 1B), and from our own experience in handling *E. paenulata* adults, it is noticeable that they emit a distinctive odour when disturbed, odour that deserves further chemical studies. Indeed, we cannot rule out the possibility that this odour is due to pyrazines, a convergent signal found in different aposematic arthropods (Woolfson & Rothschild 1990; Lindstrom *et al.* 2001). In coccinellids for instance, alkylmethoxypyrazines were tentatively identified as components of the characteristic odour of the 7-spot ladybird, *Coccinella septempunctata* L. (Moore *et al.* 1990), and later confirmed by the full characterization of 2-isopropyl-3-methoxypyrazine (Al Abassi *et al*. 1998).

Immature stages of *E. paenulata* are not so well protected against *Lycosa* spiders, albeit they also contain alkaloids (Figure 3). Most of the pupae (9 out of 10) were rejected by the spiders after the initial contact, but they later failed to molt into adults, probably as a result of injury during the attack. In the case of the larvae, our results showed that they are an acceptable food item for the spiders (7 out of 10 were consumed) (Figure 1A). The fact that inmature stages are not well protected against *Lycosa* spiders may be related to the qualitative differences between their alkaloid content and that of the adults, in particular the large amounts of alkaloids **2** and **3** that are found in the later (Figure 3).

The response of the spiders to the pupae is, nonetheless, and indication that they are not readily palatable. Moreover, half of the spiders dragged their chelicers against the sandy substrate after rejecting the pupae, suggesting a cleansing behaviour. This notion was further supported by the experiment with pupae in the field, which showed them to be well protected against ants. Pupae contain lower alkaloid loads (Table 1), which at first sight may seem contradictory for a still life instar. However, similarly to the pupae of other *Epilachna* species (Attygalle *et al.* 1993b; Schroeder *et al.* 1998a,b), *E. paenulata* pupae bear glandular hairs that secrete an oily secretion. Indeed, this oily secretion is what ants first contact *vis a vis* with an *E. paenulata* pupa, and therefore it may be responsible for the results we obtained in the tests with *L. humile* ants. In *E. borealis* and *E. varivestis*, this secretion was shown to contain azamacrolides and polyamines that are not present in other developmental stages, and are deterrent to predators. We are currently studying the pupal secretion of *E. paenulata*, which appears to be similar to that of *E. borealis* (Schroeder *et al.* 1998a,b).

Chemically, the alkaloidal defense of *E. paenulata* is similar to that of its congeners. The alkaloids vary qualitatively and quantitatively along the life span of the insects, eggs having mainly **1**, and old adults containing the four

alkaloids described. As it was the case with the Mexican bean beetle (*E. varivestis*), the alkaloid profile in older adults differs from that of younger life stages (immatures and newly-emerged adults). However, our results differ from those with *E. varivestis*, which contained primarily the homotropane **3** in the adult stage, and the pyrrolidine **4** as the main alkaloid during the juvenile stages (Proksch *et al*., 1993). Interestingly, there is an analogy between the chemistry of *E. paenulata* and *E. varivestis*, in regard to their defensive capacity. Our results showed that immature stages, that do not contain the homotropane **3,** are not well protected against *Lycosa* spiders. Similarly, the homotropane **3** was not found in recently emerged adults of *E. varivestis* (1-day old), which contrary to 7-day old *E. varivestis*, were not protected against another predatory spider (jumping spiders, *Phidippus regius*) (Eisner *et al.* 1986). Indeed, Eisner *et al.* (1986) found evidence that **3** accounts for the deterrency of *E. varivestis* blood against jumping spiders. Therefore, the absence of the homotropane **3** may explain the vulnerability of certain stages in both *Epilachna* species.

Individual quantitative analyses showed that 5-day-old adults have a higher alkaloid load (net amount and concentration) than just-emerged and older adults (10-days old) (Table 1). *E. paenulata* life cycle is completed in 70-80 days (eggs: 7 days; larvae: 21; pupae: 5-7; adults: 30-45). Adults are able to copulate the day after emergence, and females start oviposition 3 days later (Ganho & Marinoni 2000; Scatoni & Bentancourt 1999). One can speculate that it may be advantageous for *E. paenulata* to maximize its alkaloid biosynthetic capability when the oviposition rate is high, allowing for an enhanced alkaloid endowment of the eggs (Ganho & Marinoni 2000; Scatoni & Bentancourt 1999). The individual alkaloid load of *E. paenulata* adults is about five times lower that of *E. varivestis* (Proksch *et al*., 1993), which suggests that lower amounts of the alkaloids suffice for chemical protection, or that the chemical defense of *E. paenulata* includes other non-alkaloidal components that make up for the low alkaloid load.

Among the remaining stages, the maximum alkaloid concentration was found in the eggs (Table 1). Whether higher alkaloid loads confer better protection against potential predators remains unknown. Nonetheless, being motionless and highly conspicuous, it makes sense that eggs present chemical protection. Eggs show higher concentrations of **1** than adults, and virtually no **2** or **3**. One wonders if females endow their eggs differentially with the piperidine **1**, that is, if they are able to concentrate **1** in the eggs. Alternatively, the alkaloid load in the eggs may not originate only from the female. In several insects the male passes a nuptial gift to the female during copulation (Vahed 1998). Females in turn endow their eggs partially with materials from that gift (Eisner *et al.* 2002; Vahed 1998). We are currently conducting experiments with labeled alkaloid precursors to discern if this holds true for *E. paenulata*. As a final remark, we cannot exclude the possibility that embryos within eggs are able of biosynthesizing the alkaloids, as it has been questioned in other systems (Blum & Hilker, 2002). However, it appears unlikely that developing embryos will spend resources on the biosynthesis of chemical defenses that can be received from the parents.

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