Biparental Endowment of Endogenous Defensive Alkaloids in *Epilachna paenulata*

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Abstract Coccinellid beetles contain a variety of defensive alkaloids that render them unpalatable to predators. *Epilachna paenulata* (Coleoptera: Coccinellidae) is a South American ladybird beetle that feeds on plants of the Cucurbitaceae family. The defensive chemistry of *E. paenulata* has been characterized as a mixture of systemic piperidine, homotropane, and pyrrolidine alkaloids. Whole body extracts of adult beetles contain four major alkaloids: 2-(2′-oxopropyl)-6-methylpiperidine (**1**); 1-(6-methyl-2,3,4,5-tetrahydro-pyridin-2-yl)-propan-2-one (**2**); 1-methyl-9-azabicyclo[3.3.1]nonan-3-one (**3**); and 1-(2″-hydroxyethyl)-2-(12′-aminotridecyl)-pyrrolidine (**4**). Comparative studies of the defensive chemistry of eggs, larvae, pupae, and adults showed differences in alkaloid composition and concentration among life stages. While adults contained mainly the homotropane 1-methyl-9-azabicyclo[3.3.1]nonan-3-one (**3**), eggs showed the highest concentration of the piperidine 2-(2′-oxopropyl)-6-methylpiperidine (**1**). We studied the origin of this alkaloid in the eggs by feeding newly emerged, virgin adult beetles with [2-^{13}C]-labeled acetate, and by performing crosses between ^{13}C-fed and unlabeled males and females. GC-MS analysis of alkaloids from ^{13}C-fed males and females showed high incorporation of ^{13}C into the alkaloids, as evidenced from a 20–30% increase of isotopic peaks in diagnostic fragment ions, confirming the expected endogenous origin of these alkaloids. In addition, analyses of eggs from different crosses showed that labeled alkaloids from both parents are incorporated into eggs, indicating that *E. paenulata* males transfer alkaloids to the females at mating. Biparental endowment of chemical defenses into eggs has been shown previously in insects that acquire defensive compounds from dietary sources. To our knowledge, this is the first report of biparental egg endowment of endogenous defenses.

Keywords *Epilachna paenulata* · Nuptial gift · Biparental endowment · Egg defense · Coleoptera · Coccinellidae

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Introduction

Ladybirds have been a remarkable source of novel structures, mainly alkaloids (Glisan King and Meinwald 1996) and polymeric nitrogen-containing substances (Schroeder et al. 1998a, b). Such compounds, which may have a clear defensive purpose (Eisner et al. 1986, 2002; Attygalle et al. 1993a, b; Glisan King and Meinwald 1996), include azaphenalenes, azabicyclo nonanes (homotropane), aliphatic and aromatic amines, pyrrolidine and piperidine alkaloids, azamacrolides and macrocyclic polyamines. In most cases, coccinellid alkaloids are of endogenous origin (Attygalle et al. 1994; Laurent et al. 2001, 2002), although a few studies have reported ladybird species that sequester alkaloids from their food source (Witte et al. 1990; Pasteels 2007).

Epilachna paenulata Germar 1824 (Coleoptera: Coccinellidae) is a phytophagous South American ladybird beetle that feeds on the leaves of cucurbits. The adults exhibit defensive capacity against generalist predators, due to the presence of systemic alkaloids that have been characterized

as a mixture of piperidine, homotropane, and pyrrolidine alkaloids (Scheme 1). Specifically, adult beetles contain four major alkaloids: 2-(2′-oxopropyl)-6-methylpiperidine (1); 1-(6-Methyl-2,3,4,5-tetrahydro-pyridin-2-yl)-propan-2-one (2); 1-methyl-9-azabicyclo[3.3.1]nonan-3-one (3); and 1-(2″-hydroxyethyl)-2-(12′-aminotridecyl)-pyrrolidine (4) (Camarano et al. 2006). These alkaloids are present in all life stages of the insect, albeit showing qualitative and quantitative variations among developmental stages (Camarano et al. 2006).

We have shown previously that the highest alkaloid concentration in E. paenulata is found in the eggs. Indeed, the concentration of the main egg alkaloid, piperidine 1, is higher in the eggs than in the females that laid those eggs (Camarano et al. 2006), thus suggesting some mechanism of preferential endowment of alkaloid from females to eggs, or an additional source of alkaloid that finds its way into the eggs. We investigated the latter hypothesis. Specifically, we asked whether males transfer part of their alkaloids to the female, and if so, whether these alkaloids are found in the eggs.

Methods and Materials

Insects E. paenulata adults were obtained from a laboratory colony regularly maintained on squash plants (*Cucurbita* sp.), under controlled conditions of temperature (23±2°C) and a photoperiod L:D 16:8 h. For the initial settlement of the colony, individuals were collected on squash plants at organic farms near Montevideo, and new field-collected individuals were added every year. The different life stages were maintained separately in screened cages (30×30× 30 cm), and 9 to 12 food plants (3-wk-old) were replaced every 3–4 days (Camarano et al. 2006).

Alkaloid Labeling Incorporation of ^{13}C labels to the alkaloids was done by feeding the adult beetles with squash leaves treated topically with [2-^{13}C]-sodium acetate (40 mg/ml in MeOH).

To analyze a pool of individuals (N=25), complete squash leaves were treated with 3.2 μg of labeled acetate/ beetle/day. Beetles were allowed to feed for 5 days and then frozen for alkaloid analysis.

For hemolymph analyses, 80 μl of the same solution were applied on squash leaf disks (2.5 cm diam), and the disks were offered to newly emerged adults (N=8) caged in individual containers (4 cm diameter×8 cm height). Leaf disks were placed on agar (2%) to avoid dehydration and replaced daily. On day 5, the beetles were gently disturbed with forceps to trigger the reflex bleeding reaction that is typical of insects with systemic defenses, and the hemolymph was collected in glass capillaries (5 μl, Drummond Sci. Co.) for alkaloid analysis (González et al. 1999a; Camarano et al. 2006; Camarano 2008). The same protocol was followed to label the alkaloids in male or female beetles used for mating experiments and egg collection.

Control experiments were run under identical conditions for both labeling procedures, treating the leaves or leaf discs with methanol. Control and ^{13}C-fed beetles were all kept under the same environmental conditions described above.

Mating and Egg Collection Horizontal (male to female) and vertical (female to egg) alkaloid flow were studied by setting different mating pairs, in which one of the parent beetles had been fed with ^{13}C acetate (hereafter indicated with an asterisk: ♀* or ♂*).

To study male alkaloid transfer, eight virgin control females (♀) were placed individually with virgin ^{13}C-fed males (♂*). To determine if copula had taken place, the position of the beetles was recorded on video (Panasonic AG-DP200E Camera, recording 1 s every 10 min). After mating, the male was removed, and hemolymph was collected from the female 24 h later. Females were then allowed to lay eggs in the same containers (see next paragraph).

The vertical flow of alkaloids was studied similarly by collecting eggs from three groups of mating pairs: ♀* × ♂ (N=4), ♀ × ♂* (N=4), and ♀ × ♂ (N=2, control).

Alkaloid Extraction Three procedures for alkaloid extraction were carried out, depending on the matrix and sample size: (1) 25 pooled adults were killed by freezing and extracted under stirring with methanol (3 h); the extract was filtered, dried under vacuum, re-suspended in HCl (0.2 M), and washed with hexane (3×15 ml). The pH of the remaining aqueous phase was increased to 10 (NaOH, 1 M), and the alkaloids were extracted with methylene
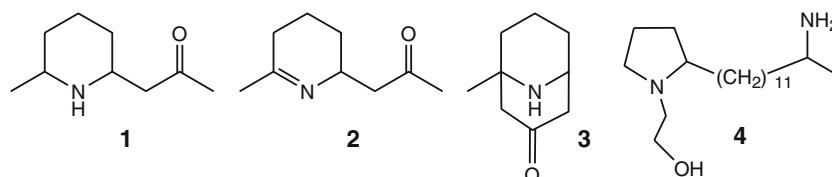
Scheme 1 Systemic alkaloids identified in E. paenulata

chloride (3×15 ml). This final extract was concentrated under vacuum, at room temperature; (2) alkaloids from the hemolymph were extracted by suspending freshly collected hemolymph in 200 µl of ammonium hydroxide (pH 8) and extracting the alkaloids with methylene chloride (3×150 µl); (3) alkaloids from the eggs were extracted with methanol (1 ml) at room temperature (24 h). Eggs were crushed to facilitate the extraction, and the solid remains were separated by centrifugation (14,000 rpm, 10 min). The supernatant solution was taken to pH 8 (NaOH), and extracted with methylene chloride (3×150 µl). In the two latter procedures, the joined methylene chloride extractions were concentrated to ca. 100 µl under N_2.

Analytical Procedures To determine ^{13}C incorporation into *E. paenulata* alkaloids, the extracts were analyzed by GC-MS as follows: extracts from pooled adults were analyzed by using an HP 5791 instrument equipped with an Elite-5 column (30 m; 0,32 mm), splitless injection in CH_2Cl_2, and a temperature program starting at 60°C (4 min) to 300°C (8 min) at 10°C/min. Injector and interphase temperature were 300°C, and total ion current detection mode was used. In the case of egg and haemolymph extracts, GC-MS analyses were performed on a Shimadzu QP 5050 or a Shimadzu QP 2010 [Carbowax 20M, 25 m, 0.32 mm; splitless injection in CH_2CI_2, 60°C (6 min) - 204°C (20 min) at 10°C/min. Injector and interphase temperature: 250°C]. Alkaloids (**1**, **2**, and **3**), where incorporation was studied, were identified previously on the basis of NMR and/or mass spectral data (Camarano et al. 2006). MS data are included in Table 1, Supplementary material. To enhance sensitivity, detection of alkaloids in the egg extracts was done by single ion monitoring (SIM) of the characteristic ions, specifically choosing the following peak clusters: 98-99-100-101-102-103 (characteristic of alkaloid **1**), 110-111-112-113-114-115-116 (characteristic of alkaloids **2** and **3**), 153-154-155-156-157-158-159-160 [cluster including M+ of **1** ($m/z = 155$) and **2** and **3** ($m/z = 153$)] (Camarano 2008). A validation process comparing mass spectra acquisition by two modes, total ion current and single ion monitoring, was carried out to ensure that acquisition by SIM had no effect on the data (Supplementary material Figs. 1 and 2).

Calculations of ^{13}C-enrichment of alkaloids were performed with freeware developed by the National Institutes of Health, US (available on line at http://sx102a.niddk.nih.gov/iso.html, last accessed in 2007, Hess et al. 2002). The program compares the relative intensities of all ion peaks within an ion cluster, for enriched and control samples, and calculates the enrichment as percent MPE (mole percentage excess) of the stable isotope related to its natural abundance, as well as the maximum number of labels incorporated.

Results

[2-^{13}C]-Acetate Incorporation into E. paenulata Alkaloids The analysis of alkaloid extracts from pooled adults ($N=25$) showed only the homotropane **3**. Analysis of the corresponding peak clusters (m/z 110 and 153) showed an incorporation of ^{13}C from acetate into this alkaloid, with a MPE of 22%.

In hemolymph samples, despite their low concentration, alkaloids **1**, **2**, and **3** were detected by GC-MS analyses in all the samples (e.g., alkaloid **3**, Fig. 1). The relative intensities within different ion clusters showed that *E. paenulata* incorporated labeled acetate into the three defensive alkaloids, with MPE of 20% to 30% (Table 1, Fig. 1). Noteworthy, this methodology allows the follow-up of ^{13}C incorporation within an individual through time, since hemolymph extraction does not injure the insect.

Alkaloid Egg Endowment (Vertical Flow) GC-MS analyses of egg samples showed detectable amounts of alkaloids **1** and **3** (SIM detection). Extracts of eggs laid by females from ♀* × ♂ mating pairs evidenced ^{13}C incorporation (Table 2) with up to five labels in alkaloid **3**, indicating that females transfer their own alkaloids to the eggs. Interestingly, the analyses of eggs laid by control females mated to ^{13}C-fed males (♀ × ♂*) also showed labeled alkaloids, indicating that male alkaloids are also transferred to the eggs they sire (Table 2, Fig. 2).

Male Nuptial Gift (Horizontal Flow) The existence of an alkaloidal nuptial gift from males to females at mating can be inferred from the fact that male alkaloid finds its way into the eggs. We also studied the fate of the male alkaloids in the females, by analyzing the hemolymph of mated females from ♀ × ♂* mating pairs. The main alkaloids in these hemolymph samples were **2** and **3**, but no evidence of ^{13}C labeling was found, suggesting that the male alkaloids do not pass the systemic circulation in the female, but rather remain confined to the female reproductive tract.

Discussion

The three major alkaloids (**1-3**) in *E. paenulata* showed incorporation of [2-^{13}C]-acetate when fed with squash leaves treated with the labeled precursor. These results confirm the notion that defensive alkaloids in this beetle are endogenous, as it is the case in other coccinellid species (Attygalle et al. 1993b, 1994, 1999; Glisan King and Meinwald 1996; Farmer et al. 1997; Radford et al. 1997; Schroeder et al. 1998a, b; Braekman et al. 1999; Laurent et

Fig. 1 MS trace of alkaloid **3** from hemolymph samples of *E. paenulata* individuals fed on normal diet (**a**) and on [2-^{13}C]-sodium acetate enriched diet (**b**). Two characteristic ion clusters show evidence for ^{13}C incorporation, given the relative increase of ions up to M+4. The relative intensities are presented in normalized form in **c** (*m/z* 153 cluster) and **d** *m/z* 110 cluster (*filled squares* control; *empty squares* ^{13}C-enriched samples) (TIC detection)
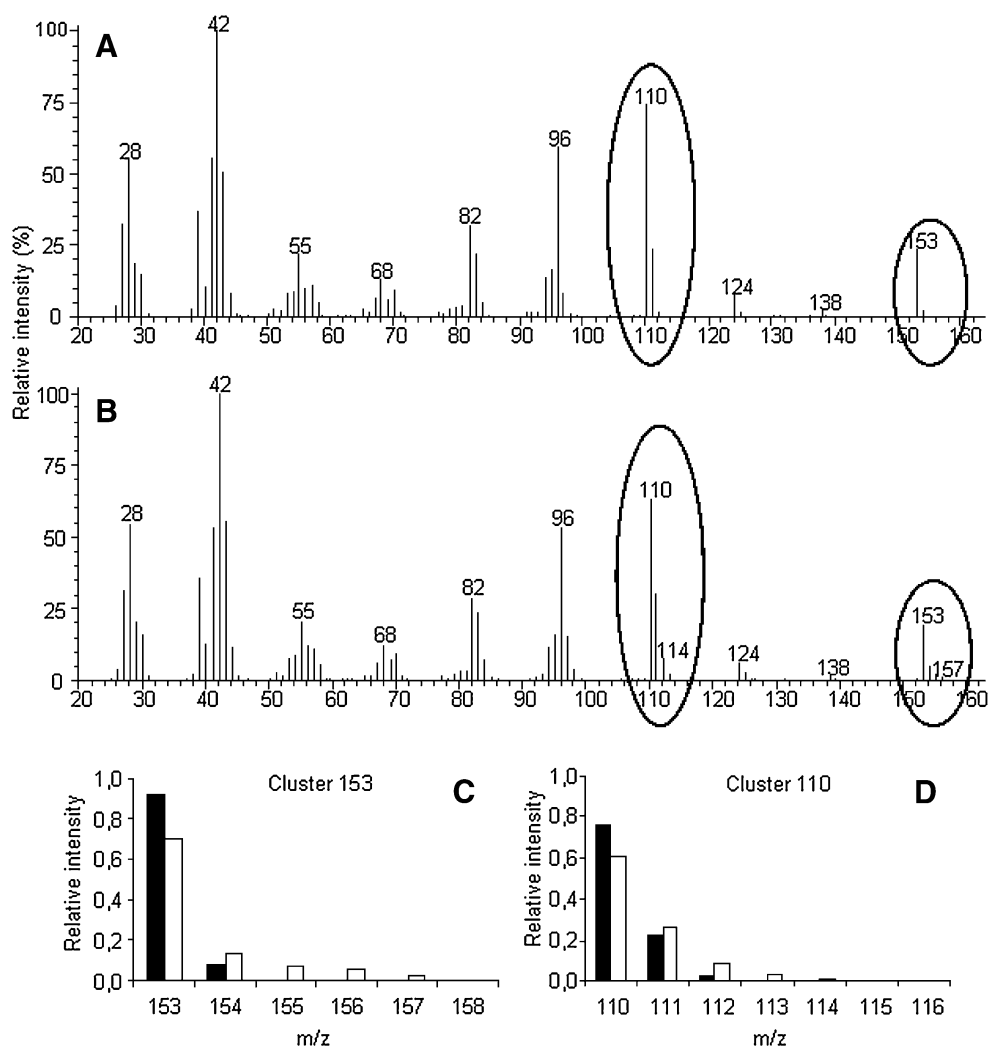
al. 2001, 2002). When using SIM detection, our data show that the alkaloids incorporated up to five acetate units (Table 2), which is in accordance with the polyacetate biosynthetic pathway that has been proposed for coccinellid alkaloids (Laurent et al. 2001). Preliminary experiments also showed weak incorporation of [18-D$_3$]-stearic acid into *E. paenulata*'s piperidine **2** (data not shown), suggesting a fatty acid-derived pathway. This is in agreement with earlier findings by Laurent et al. (2002), who demonstrated a fatty-acid biosynthetic pathway for the alkaloids adaline and coccinelline in *Adalia 2-punctata* and *Coccinella 7-punctata*, respectively. In plants, pyrrolidine and piperidine alkaloids are derived from amino acids such as ornithine and lysine, which cannot be ruled out from our data (Dewick 1997).

The incorporation of ^{13}C into the alkaloids allowed us to study the horizontal and vertical flow of chemical defenses in this beetle, since it made it possible to differentiate alkaloids from males and females. Eggs coming from one "labeled" parent, either female or male, showed alkaloid **1** and **3** labeled with ^{13}C (Table 2). Although egg protection

by female defensive compounds is expected, our data show that males also can protect the eggs that they sire, by transferring part of their alkaloidal load to the females at mating. Since the males were removed from the mating container several days before the eggs were laid, the male must transfer the alkaloids to the female, and she then transfers the male's and her own alkaloids to the eggs.

Table 1 Enrichment of *E. paenulata* alkaloids in hemolymph samples from adults fed with [2-^{13}C]-sodium acetate

Alkaloid	*m/z*	Max. number of labels	Percent MPE[a] (mole percentage excess)
1[b]	98	1	26±5
2	110	2	26±9
	153	2	24±10
3	110	3	23±7
	153	4	24±7

[a] Data are shown as mean±SD (*N*=8) of the % MPE
[b] For alkaloid **1**, results are presented for ion cluster *m/z* 98, due to the low intensity of the M$^+$ cluster (*m/z* 155)

Table 2 Enrichment and maximum number of labels incorporated to alkaloids **1** and **3** in *E. paenulata* egg samples from different mating pairs

Alkaloid	m/z cluster	Origin of egg sample			
		♀* x ♂		♀ x ♂*	
		Max. number of labels	Percent MPE	Max. number of labels	Percent MPE
1	98	3	16±4	3	18±7
3	110	3	28±5	3	23±6
	153	5	23±5	3	20±5

Results are shown as mean±SD (*N*=4 for both mating types)

* Denotes individuals fed on [2-^{13}C]-sodium acetate enriched diet

Insect eggs often contain chemical defenses, and these toxins are provided either by the female, the male, or both sires (Hinton 1981; Hilker 1994). Males of several insect species pass a nuptial gift to the female at mating (Vahed 1998, 2007), and it is not uncommon that part of this gift is allocated to the eggs (Vahed 1998; Eisner et al. 2002). Biparental endowment of egg defenses has been demonstrated in a number of systems in which the defensive chemicals are acquired from the diet (Ferguson et al. 1985; Dussourd et al. 1988; Nishida and Fukami 1989; Blum 1992; González et al. 1999a; Hartmann 1999; Eisner et al. 2002). To our knowledge, however, biparental egg endowment of endogenous defenses, as shown here, has not been demonstrated before. In the case of cantharidin, a terpene anhydride produced de novo by some oedemerid and meloid beetles, the situation varies among species. In

Oedemera femorata (Coleoptera: Oedemeridae), both sexes produce cantharidin, but females produce higher amounts than males. Transfer of cantharidin from males to females at mating was not found in this species, and hence, cantharidin in the eggs appears to originate solely from females (Carrel et al. 1986; Holz et al. 1994). In members of Meloidae, such as *Lytta vesicatoria*, the females are unable to produce their own cantharidin, and they receive it as a nuptial gift from males at mating. Females then bestow part of this cantharidin gift into the eggs (Meyer et al. 1968; Schlatter et al. 1968; Sierra et al. 1976; Eisner et al. 2002). Conversely, in the case of *Epicauta funebris*, only adult males keep the larval capability of biosynthesizing cantharidin, and although there is seminal transmission of cantharidin, it is not transferred into eggs, but rather kept by the females, likely for self-protection (McCormick and Carrel 1987; Carrel et al. 1993).

In *E. paenulata*, the presence of male alkaloid in the eggs indicates a nuptial gift, but the pathway by which this gift reaches the eggs remains unknown, since we did not detect male-labeled alkaloid in the hemolymph of the females they mated with. This result suggests that most of the male alkaloid remains confined within the female reproductive tract, from where it is allocated into the eggs, probably as they are laid. Careful dissections of the female reproductive tract and analysis of its parts, in particular the spermatheca (Rossini et al. 2001; Nikbakhtzadeh et al. 2007), should provide insight about the fate of male alkaloids after seminal transfer. In other insects, defensive male nuptial gifts become systemic within the female body. In *Utetheisa ornatrix* (Lepidoptera: Tortricidae), for exam-
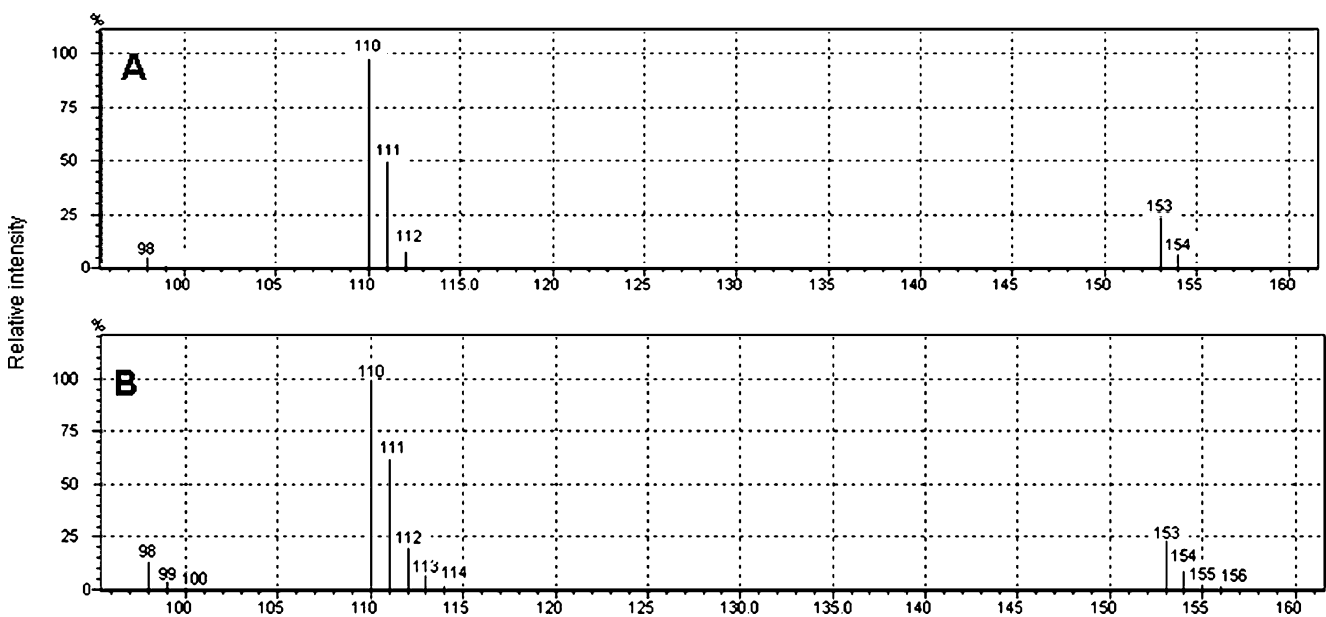
Fig. 2 MS traces of homotropane **3** from eggs laid by control *E. paenulata* females (**a**) and control *E. paenulata* females mated to ^{13}C-fed males (**b**) (SIM detection)

ple, females can use the pyrrolizidine alkaloids transferred by males both for their own defense and for the protection of the eggs (González et al. 1999b), since the alkaloids become systemic even before the mating pair uncouples.

The adaptive value of male egg protection in *E. paenulata* is open to question. The female load may be insufficient to properly protect the eggs against predators and/or microorganisms. Obtaining and testing eggs with different alkaloidal loads, below and above female levels, would be useful to test this hypothesis. If male defenses do make a difference for the fitness of the female, it opens the possibility for some overt or cryptic process of sexual selection that may favor males with larger alkaloid loads.

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