

Running head: Hairpencil secretion of butterfly

**Major components in the hairpencil secretion of a butterfly,
Euploea mulciber (Lepidoptera, Danaidae): Their origins
and male behavioral responses to pyrrolizidine alkaloids**

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Abstract

Two compounds, 9,10-epoxytetrahydroedulan (ET) and viridiflorine β -lactone (VL), were identified as major components from the hairpencils of field-caught males of a danaid butterfly, *Euploea mulciber*. By contrast, laboratory-reared males entirely lacked VL, but possessed a significant quantity of ET. Various feeding experiments with larvae and indoor adult males strongly suggested that ET is biosynthesized de novo only after eclosion from nutrients ingested during the larval development. Since VL was suspected to be derived from pyrrolizidine alkaloids (PAs) acquired as an adult, tests for feeding response to and oral administration of four PAs (a 4:1 mixture of intermedine/lycopsamine, heliotrine, monocrotaline, and retronecine) were conducted. When the tarsi or proboscis were stimulated with PA solutions, males showed positive feeding responses (proboscis extension and sucking movements) to intermedine/lycopsamine, heliotrine, and retronecine in decreasing order of responsiveness, thereby providing evidence that male adults are endowed with taste receptor(s) specific to PAs on the legs as well as on the proboscis. Differently from gustatory responsiveness, only males fed with intermedine/lycopsamine produced a significant quantity of VL (ca. 35 $\mu\text{g}/\text{male}$), whereas those that ingested heliotrine or monocrotaline hydrochloride produced traces of VL ($<0.18 \mu\text{g}/\text{male}$). Uptake of retronecine did not lead to VL formation at all. In behavioral bioassays to test the attractivity of PAs to males, all individuals tested were attracted exclusively to

intermediate/lycopsamine. This shows that certain PA(s) per se serve as attractant(s) for males in locating PA sources, and further suggests that in the field, males will seek particular PA(s) that are indispensable as precursors for the efficient biosynthesis of VL.

Keywords: Biosynthesis; Sex pheromone; Pyrrolizidine alkaloids; Attractivity; Gustatory reception

1. Introduction

Chemical signaling by males is no less important than by females in the courtship of some lepidopterans (e.g. Eisner and Meinwald, 1987; Birch et al., 1990). In butterflies, volatile chemicals disseminated by males from the androconial organs have been reported to generally play a much more significant role than those of females during the sequence of mating behavior (Boppré, 1984; Honda, 2005). Among the most conspicuous androconial architectures butterflies possess are the scent-producing organs found in the family Danaidae. Male adults of most danaids have two types of scent-producing organs, i.e. a pair of abdominal hairbrushes (hairpencils) and alar patch- or pouch-like glands (sex brand) (Pliske and Salpeter, 1971; Boppré and Vane-Wright, 1989). The hairpencils, though ordinarily retracted inside the abdomen, are

extruded and splayed nearby the female at a species-specifically programmed timing during the precopulatory pursuit or hovering. The hairpencil chemicals are perceived by females at the time of hairpencilling (Brower et al., 1965; Motofuji, 1976).

Extensive investigations conducted of a number of lepidopterans have revealed the occurrence of diverse compounds in the androconial secretions, which are thought to be highly responsible for sexual communication. Dihydropyrrolizine derivatives such as danaidone, hydroxydanaidal, and danaidal are the components most frequently encountered in danaid secretions (e.g. Meinwald et al., 1966, 1974; Edgar et al., 1971, 1973; Komae et al., 1982; Honda et al., 1995; Schulz, 1998; Schulz et al., 2004). In some danaids, these constituents are accompanied by terpenoids, aliphatic acids, esters, and other miscellaneous compounds (Bellas et al., 1974; Schulz et al., 1988a,b, 1993b; Schulz and Nishida, 1996), thus probably furnishing a species-specific bouquet that serves as an arrestant or an aphrodisiac eliciting the receptive response from females (Meinwald et al., 1969; Pliske and Eisner, 1969; Nishida et al., 1996).

Close associations of some lepidopteran taxa with pyrrolizidine alkaloids (PAs) have long drawn attention and have been well documented in regard to pharmacophagy (Boppré, 1990; Hartmann and Ober, 2000). For example, larvae of several moth species in the family Arctiidae are known to take up and store PAs from PA-containing host plants (Weller et al., 1999) and adults, particularly males, of most

butterfly species of the Danaidae and Ithomiidae also congregate on decaying or dead plants containing PAs in the families Boraginaceae, Asteraceae, and Fabaceae, from which they imbibe the alkaloids (Pliske, 1975a; Boppré, 1981). PAs are also incidentally imbibed together with the nectar of PA-containing inflorescence (Pliske, 1975b). Plant-acquired PAs are sequestered, often chemically modified, and systemically stored (e.g. Edgar and Culvenor, 1974; Trigo et al., 1994, 1996; Brückmann et al., 2000; Hartmann et al., 2005). In Lepidoptera, ingested PAs have been shown to be utilized as precursors for pheromone biosynthesis and also for chemical protection against predators (Boppré and Schneider, 1985; Orr et al., 1996; Conner et al., 2000; Rossini et al., 2003). The regulatory action of PAs on the development of coremata (tubular abdominal scent organs of arctiids) has also been reported for arctiid moths (Boppré and Schneider, 1985; Egelhaaf et al., 1992). Another PA-insect relationship is found in the egg-laying by a primitive Old World danaid butterfly, *Idea leuconoe*, which exploits macrocyclic PAs present in its host plant (*Parsonsia laevigata*) as oviposition stimulants (Honda et al., 1997).

It is fully established that hydroxydanaidal, an important male pheromone of some arctiid moths, is biosynthesized from various PA precursors through specific chemical transformations (Schulz et al., 1993a; Hartmann et al., 2003, 2005). In butterflies, however, very little is known about the biosynthetic mechanisms involved in the production of androconial substances including dihydropyrrolizines.

Since earlier work has reported that in *Danaus* butterflies, danaidone was derived from PAs (Schneider et al., 1975), it seems to be a general notion that danaid males are capable of handling a wide variety of PAs to produce dihydropyrrolizine derivatives. Recently, however, we have demonstrated that males of *Parantica sita*, an Asian danaid butterfly, can only successfully convert limited chemical types of PAs into danaidone (Honda et al., 2005). Apart from the derivation of androconial substances, only a few studies have experimentally substantiated their pheromonal activity. Therefore, the pheromone system of danaid butterflies, on the whole, remains poorly understood.

We chose as a research object the striped blue crow butterfly, *Euploea mulciber* (Lepidoptera, Danaidae), which belongs to the same subtribe Euploeina as does *I. leuconoe* (Ackery and Vane-Wright, 1984). The genus *Euploea* is a relatively large group comprising more than 50 species. *E. mulciber*, a subtropical or tropical butterfly occurring from the Southwestern Islands of Japan to Indo-Australian regions, utilizes as host plants a great variety of species in the families Moraceae, Apocynaceae, and Asclepiadaceae. Their major host plants in Japan are *Ficus microcarpa*, *F. benjamina* (Moraceae), and *Nerium indicum* (Apocynaceae). Another milkweed, *Asclepias curassavica* (Asclepiadaceae), is also a food plant very suitable for the larval growth. All these plants are devoid of PAs. Presumably for that reason, male adults of the butterfly as well as other danuids are often observed in the field to visit flowers and withered or diseased tissue of PA-containing

plants. *E. mulciber* males, unlike *Danaus* males, never exhibit contact behavior between the two scent organs prior to courtship, which, in certain danaid species, is believed to be a prerequisite for the production of androconial substances (Brower and Jones, 1965; Boppré et al., 1978). Although Francke et al. (1989) have already reported a terpenoid component (9,10-epoxytetrahydroedulan) from the hairpencils of *E. mulciber* and two other *Euploea* species (*E. klugii* and *E. leucostictos*), almost no information is available on other constituents, their origins, or about how butterflies manipulate plant-acquired PAs.

In the present work, we investigated volatile compounds in the hairpencils of *E. mulciber* and attempted to elucidate the derivation of the major components. Furthermore, the attractivity of and gustatory response to several PAs in adult males were also examined by behavioral bioassays.

2. Material and methods

2.1. Insects

E. mulciber adults captured in the Southwestern Islands (Okinawa and Kagoshima prefectures, Japan) and their progeny were used in this study. Larvae fed with fresh leaves of *F. microcarpa*, *N. indicum* or *A. curassavica* were raised under standard laboratory conditions (16L:8D, 24-26 °C). Occasionally, larval rearing was conducted on potted plants in a greenhouse (23-27 °C and under a natural photoperiod regime).

Adults were sexed immediately after eclosion and males were kept in transparent plastic chambers at 25 °C under a 16L:8D regime (ca. 100 lux). Throughout the experiments, they were fed with 15% aq. sucrose solution once a day, and exposed daily to incandescent light (ca. 3500 lux) for at least 8h during the photophase, unless otherwise noted. To maintain laboratory cultures, several young adults of both sexes were released into an outdoor cage equipped with flowers and PA-containing plants (*Eupatorium* spp.) and allowed to mate. After copulation, gravid females were transferred to the laboratory to collect eggs.

2.2. Preparation of hairpencil extracts and chemical analysis

The hairpencils were artificially protruded with forceps, excised, soaked individually in 100 µl of purified dichloromethane, and stored at -20° C until use. For laboratory-reared males, the hairpencils were dissected on a predetermined day after emergence. The hairpencil extracts of wild males, captured in October, 2001 and 2005, were prepared within 24 h of collection.

Chemical analyses of hairpencil extracts were performed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). GC analyses were carried out on a Shimadzu GC-14A gas chromatograph equipped with a flame-ionization detector, using a EQUITY-1 fused-silica capillary column (Supelco, 0.25 mm I.D. x 15 m, 0.25 µm film thickness). Samples were injected splitless at 250°C using N₂ as the carrier gas (flow; 1 ml/min) and the oven temperature

was programmed from 50°C (held initially for 5 min) to 300°C (held for 10 min) at 10°C/min. EI-MS spectra were recorded at 70 eV on a Shimadzu GCMS-QP5000 mass spectrometer coupled with a Shimadzu GC-17A gas chromatograph, using a DB-1 fused-silica capillary column (J&W, 0.25 mm I.D. x 15 m, 0.25 µm film thickness) and He as the carrier gas (flow; 1 ml/min). The GC system was operated with the same temperature program as that employed in GC analyses. Identification of the components was based on a comparison of their GC retention data and mass spectra with those of authentic samples or reported data.

Quantification of the components was performed by GC using benzyl alcohol as the internal standard. The amount of each component was estimated from its peak area in terms of benzyl alcohol.

2.3. PAs

Heliotrine (Latoxan, purity: 98%) and monocrotaline (Aldrich, 99%) were commercially available. Since monocrotaline was sparingly soluble in water, its HCl salt (monocrotaline hydrochloride) was prepared when necessary. Retronecine (98% by GC and NMR) was prepared from monocrotaline by hydrolysis with aq. NaOH. A PA mixture composed of intermedine and lycopsamine (ca. 4:1) was obtained from a methanol extract of the underground part of *Eupatorium chinense* ssp. *sachalinense* (Asteraceae) collected in Hiroshima prefecture, and purified by ion-exchange chromatography on Accell Plus

CM and QMA resins (Waters). Their structures, ratio, and purity (ca. 95% as a whole) were determined by GC-MS and ^1H - and ^{13}C -NMR analyses (Roeder, 1990; Logie et al., 1994). NMR spectra were measured in D_2O with a JEOL JNM-A400 FT-NMR spectrometer with tetramethylsilane as the external standard. The chemical structures of these PAs are shown in Fig. 1.

2.4. Feeding response to PAs

Seven-day-old males ($N=10$) were tested by a similar method of Honda et al. (2005). Before the bioassay, each individual was given water and 15% aq. sucrose to satiety. This experiment was designed to examine the elicitation of a sequential behavior that consisted of two phases; proboscis extension by stimulation of tarsi with PA solutions and actual feeding on them. An individual was placed on a filter paper strip (20 x 20 mm) impregnated with a 2.5% w/v (net concentration of PA) aq. solution of a PA. First, the number of individuals that spontaneously uncoiled and extended the proboscis within 5 s after tarsal contact with a treated paper was recorded. If an individual was not induced to uncoil the proboscis by tarsal stimulation, 1 μl of the same solution was applied with a microsyringe to the coiled proboscis. Finally, the number of individuals that exhibited a positive feeding behavior comprising proboscis extension and sucking for more than 30 s was recorded. Unresponsiveness of males to water was confirmed just before each trial. The same test was replicated with other older males

(26 to 40 days old, $N=10$) to examine the age dependence of responsiveness. The response was expressed as the percentage of individuals that responded positively.

2.5. Oral administration of PAs

After 7 days of eclosion, individual males were fed daily with 0.2 mg (net weight of PA) of any one of the four PA samples for 7 days (1.4 mg in total for each male). For PA samples that hardly stimulated voluntary feeding, these were given to males together with 15% sucrose solution. Control males were fed with plain sucrose solution only. On day 14 of emergence, the hairpencils were dissected individually and extracted as described above.

In addition to PA-administration experiments, chemical analysis was also conducted of the hairpencil secretion of male adults derived from larvae that had been reared on *P. laevigata*, the one host plant of *I. leuconoe*, during the last (5th) larval instar. Since this plant has been reported to contain macrocyclic PAs (e.g. Abe and Yamauchi, 1987; Abe et al., 1991), this experiment was aimed to test whether or not the butterfly can utilize PAs acquired during the larval stage. Although most larvae failed to perform well on this non-host plant, several larvae developed into adults. These males were fed with 15% sucrose solution alone for 14 days.

2.6. Investigation of the origin of 9,10-epoxytetrahydroedulan

To examine whether 9,10-epoxytetrahydroedulan was biosynthesized de novo or derived from secondary metabolite(s) taken up as larvae from the host plants, 3 groups of larvae were reared on any one of the food plants, *F. microcarpa*, *N. indicum* and *A. curassavica*, and the males thereof were fed daily with either 15% aq. sucrose or water alone for 14 days after eclosion. Those that were given water alone were always kept in dim light until use so as to repress their energy consumption to a low level. Chemical constituents of the above 3 plants were also examined for the presence of 9,10-epoxytetrahydroedulan and its possible precursor, dihydroedulan: Fresh leaves (10g) of each plant were extracted with isopentane (200 ml) below 15°C for 1 week. The extract was filtered and concentrated in vacuo at R.T. to ca. 1 ml. After removing the precipitates (solid hydrocarbons) by centrifugation and adding 1 ml of dichloromethane to the supernatant, the resulting clear solution was subjected to GC and GC-MS analyses under similar operational conditions to those used for the analysis of hairpencil extracts.

2.7. Age dependence of 9,10-epoxytetrahydroedulan formation

Individual larvae were reared on one or two of the three food plants described above. After eclosion, male adults were fed daily with 15% sucrose solution until dissection. On post-emergence days 0 (within 12 h), 3, 7, 11, 14, 20, and 30, the amount of 9,10-epoxytetrahydroedulan produced in the hairpencils was determined.

2.8. Attraction test to PAs

The attractivity to adult males of 4 PAs, i.e. intermedine/lycopsamine, heliotrine, monocrotaline, and retronecine, was tested at 25°C in the laboratory with good ventilation. A methanol solution of a PA was uniformly applied to a filter paper disc (4 cm in diameter) so that the PA concentration was adjusted at 410 nmol/cm². Four paper discs treated with different PAs and a control disc of the same size treated with an equal amount of the solvent alone were held by a wire arm at 1 m high from the floor and placed at regular intervals (about 30 cm apart from each other) on a circle 60 cm across in the experimental arena (150 x 70 cm). The floor of the arena was covered with green plastic plates to imitate green vegetation. After evaporating the solvent, each disc was moistened by spraying water to facilitate PA vaporization. Subsequently, 10 naïve males of 14 to 34 days old that had never had an access to PAs were released and allowed free flight over the arena under illumination with fluorescent lamps (3500 lux). Three h before the bioassay, males were fed with water and 15% aq. sucrose to satiety. The disc that received the first visit followed by proboscis extension and sucking movements by each male during a 30-min assay period was recorded.

3. Results

3.1. Identification of major hairpencil volatiles of wild males

Representative gas chromatogram of a hairpencil extract from a field-caught male is shown in Fig. 2 (A). Two major components, A1 and A2, were detected. Compound A1, on GC-MS, showed diagnostic fragment ions at m/z 100 (18%), 85 (23), 72 (12), 71 (21), 67 (15), 57 (79), 55 (18), 45 (49), 43 (100), and 41 (32). Although its molecular ion was not observed, the fragmentation pattern of the compound was quite similar to that of viridiflorine β -lactone, which has previously been identified from the hairpencils of another danaid butterfly, *I. leuconoe* (Nishida et al., 1996). The mass spectrum and GC retention time of A1 coincided well with those of the β -lactone of viridifloric acid, one of the two possible lactones that can be prepared by the reaction between methanesulfonyl chloride and necic acids [(+)-trachelanthic and (-)-viridifloric] (Schulz and Nishida, 1996) derived from intermedine/lycopsamine (**1**) by alkaline hydrolysis. The identity was further corroborated by comparison with the reported component present in a hairpencil extract of *I. leuconoe*. Accordingly, compound A1 was identified as viridiflorine β -lactone [(2*S*,3*S*)- or (2*R*,3*R*)-2-hydroxy-2-isopropylbutano-3-lactone, hereafter abbreviated VL]. This compound has 2 chiral centers at C2 and C3 positions, however, their absolute configurations remained undetermined.

Peak A2, giving fragment ions at m/z 195 (8%), 177 (8), 153 (38), 135 (27), 123 (20), 111 (26), 95 (34), 83 (28), 69 (46), 55 (51), and 43 (100), was assumed to be 9,10-epoxytetrahydroedulan, which has already

been isolated and identified from some *Euploea* species including *E. mulciber* (Francke et al., 1989). When measured at 25 eV, the compound gave a weak M^+ at m/z 210 (5%), of which molecular formula was definitely determined, by exact mass measurement (high-resolution MS), to be $C_{13}H_{22}O_2$ (Calcd. for $C_{13}H_{22}O_2$, 210.16198; Obsd. 210.16555). Compound A2 was, thus, concluded to be 9,10-epoxytetrahydroedulan (ET). The chemical structures of the 2 identified compounds are shown in Fig. 3.

3.2. Feeding response of laboratory-reared males to PAs

Both intermedine/lycopsamine (**1**) and heliotrine (**2**) strongly evoked feeding (100% response) as shown in Fig. 4. Several individuals spontaneously uncoiled and extended the proboscis seeking for PAs immediately after contacting some of the PAs with mid- and hind-legs. In the experiment with 7-day-old males (Fig. 4A), the proportion of individuals that exhibited proboscis extension upon tarsal contact with compounds **1** and **2** were 70% and 10%, respectively. In contrast, no males responded positively to HCl salt of monocrotaline (**3**), while retronecine (**4**) elicited an intermediate feeding response (60%), although tarsal contact did not stimulate proboscis extension. All those that extended the proboscis eventually sucked PA solutions.

The older males (Fig. 4B) also showed a similar responsive profile for the 4 PAs, but proboscis extension appeared to be more readily triggered by tarsal contact with PAs (100%, 50%, and 10% for

compounds **1**, **2**, and **4**, respectively). However, compound **3** altogether failed to elicit any feeding response.

3.3. Effect of uptake of PAs and their structures on the chemical composition of the hairpencil secretion

Representative gas chromatograms of hairpencil extracts prepared from males that ingested compound **1** (B) and a control male (C) are shown in Fig. 2. All control males secreted ET, but lacked a peak corresponding to VL, whereas those that ingested **1** possessed both ET and VL. Furthermore, Fig. 5 clearly indicates that males of every group, regardless of the treatments, secreted ET at averages ranging from 2.4 to 9.2 µg/male. This suggests that *E. mulciber* males have more or less the intrinsic ability to produce ET by themselves. On the other hand, a significant quantity (35 µg/male on average) of VL was produced when males fed on compound **1**. However, the uptake of **2** or **3** resulted in the formation of very small amounts of VL (0.18 and 0.09 µg/male, respectively) and no trace of VL was detected from the males that were given **4**. Males that originated from larvae raised on a PA-containing plant, *P. laevigata*, also produced VL at an average of 5.4 µg/male. This indicates that certain PAs contained in the larval diet were efficiently incorporated and converted to VL.

In addition to ET and VL, some individuals of both field-collected and PA-fed males were found to have small amounts of danaidone and/or hydroxydanaidal, which are well-known dihydropyrrolizines present in

the androconial secretions of many danaids. The rough estimates of per capita quantities of danaidone and hydroxydanaidal found in the hairpencils, as determined as described previously (Honda et al., 1995), ranged from 0 to 0.31 and from 0 to 9.1 μg , respectively. Apparently, not all individuals that actually had or were suspected to have had access to PAs possessed these compounds, while control males produced neither of them. All males, independent of their feeding history, had in common small quantities of other miscellaneous compounds that were considered to be ubiquitous hydrocarbons, esters, and aliphatic acids, but they were not pursued further.

3.4. Effect of larval and adult diets on the production of 9,10-epoxytetrahydroedulan and the age dependence of its quantity

Males of every cohort, irrespective of their larval diets, produced similar amounts (5.3-5.9 μg /individual) of ET (Fig. 6). Since even the males that had been given water only ever since their emergence were able to produce ET in an amount comparable to those found in males fed with aq. sucrose, it is evident that adult diet is not essential for ET formation. These findings suggested that certain secondary metabolites or nutrients acquired from food plants during the larval development were directly or indirectly responsible for ET formation.

Then, we examined volatile components of 3 food plants, *F. microcarpa*, *N. indicum*, and *A. curassavica*, to ascertain whether or not 9,10-epoxytetrahydroedulan or dihydroedulan was contained in these

plants. However, neither compound was detected from any of the plants.

Males fresh from eclosion (day 0) were found to have an extremely small amount of ET (0.08 $\mu\text{g}/\text{male}$)(Fig. 7). However, its quantity gradually increased with age, reaching a maximum at an average of 5.3 $\mu\text{g}/\text{male}$ on day 14 and thereafter showed a downward tendency, turning back to the level of day 7 after 30 days. Surprisingly, 60-day-old males ($N=3$) still retained nearly the same quantity of ET (3.5 $\mu\text{g}/\text{male}$) as that possessed by 30-day-old males.

3.5. Attractivity of PAs to male adults

As shown in Fig. 8, all males tested were attracted exclusively to compound **1**, whereas the other PAs did not receive even temporary visits. Each male alighted on disc **1** after short-duration aerial searching with no sign of confusion. This indicates that males are endowed with a refined olfactory sense to detect and recognize particular PA(s) they prefer.

4. Discussion

4.1. Components of the hairpencil secretion of *E. mulciber*

To the best of our knowledge, ET is the sole component so far reported from the hairpencils of *E. mulciber* (Francke et al., 1989). We reconfirmed its occurrence as one of the major components in the

hairpencil secretion. In this study, we found an additional dominant component, VL, from wild males. This compound might have possibly been missed in the earlier study, but we think that this inconsistency arose from the difference in age of individual males examined, because VL was detected only from males that ingested PAs (Fig. 5) and our observations in an outdoor cage revealed that males usually began to seek for and feed on PAs several days after emergence. VL is a peculiar compound with a β -lactone structure, which was identified for the first time from the hairpencils of another danaid butterfly, *I. leuconoe*, as one of the male sex pheromones (Nishida et al., 1996). A recent paper also reports on its presence in the alar fringes of a few ithomiid butterflies (Schulz et al., 2004). Although the stereochemistry of VL produced by *E. mulciber* is unknown, the compound most likely has (2*S*,3*S*)-configuration, because both intermedine and lycopsamine have *S*-configuration at C2' (see discussion below) and *I. leuconoe* secretes (2*S*,3*S*)-VL (Schulz and Nishida, 1996). That *E. mulciber* and *I. leuconoe* share VL as a predominant component is congruent with their close phylogenetic relationship (subtribe Euploeina) argued by Ackery and Vane-Wright (1984). However, no reports have ever referred to the presence of ET in the secretion of *I. leuconoe*. To date, danaid hairpencil secretions have been examined for more than 30 species covering most genera of the family (see references cited). Since ET has only been found from *Euploea* species (Francke et al., 1989) and is also suspected to be present

in other *Euploea* species (Edgar, 1975, 1982), this compound appears to be specific to the genus *Euploea*.

4.2. Differential feeding response to PAs

Males showed strong feeding responses to compounds **1** and **2**, and engorged them (Fig. 4). This coincides well with previous reports that PAs act as phagostimulants (Pliske, 1975a; Pliske et al., 1976). However, all PAs did not stimulate feeding by males. While compound **4** elicited a moderate response, compound **3**, a diester-type PA, did not at all. This means that certain PA(s) may be inactive to gustatory sense, or perhaps even act as antifeedants, because males seemed somewhat unwilling to imbibe a sucrose solution admixed with **3**. As compared with males of another danaid, *P. sita*, which exhibited a very strict feeding behavior, positively responding to **1** only (Honda et al., 2005), *E. mulciber* males appear to have a broader spectrum of gustatory responsiveness to PAs. In contrast to these danaids, males of *Danaus gilippus* and some arctiid moths voluntarily imbibe monocrotaline (**3**) (e.g. Eisner and Meinwald, 1987; Conner et al., 2000).

Butterflies have been shown to possess gustatory sensilla on the tarsi that sensitively respond to sugars (Chapman, 1995). Immediately when the mid- and hind-legs (the stub-like forelegs are normally held tightly to the body) were brought into contact with a solution of **1**, most males were observed to spontaneously uncoil and extend the proboscis seeking for the PA. Compound **2** also released similar behavioral

responses from several males. Since no individuals, even at close range, tried uncoiling the proboscis as long as their legs were kept out of contact with PA solutions, we think that there seems no possibility that the proboscis extension response was evoked by some olfactory stimuli, although olfaction would profoundly be involved in their attraction to PAs (see 4.5). Therefore, these results provide the first evidence that male adults possess chemotactile (gustatory) receptor(s) specifically responsive to PAs on the mid- and/or hind-legs as well as on the proboscis. Larvae of some arctiids have been shown to possess specific taste receptors for PAs (e.g. Bernays et al., 2002, 2004). Although PA responsiveness of the proboscis did not seem to vary with age, that of the tarsi appeared to be enhanced as males got older (Fig. 4B). We hypothesize that this phenomenon was caused by PA-deficit stress which augmented their appetite for PAs.

4.3. Selective utilization of particular PA(s) for the production of VL

Since PA-unfed (control) males entirely failed to produce VL, it is obvious that they need to ingest some special compound(s) other than nutrients to biosynthesize VL. Of the 4 PAs administered, only compound **1** served as a significant precursor for VL biosynthesis, though **2** and **3** also afforded VL in traces (Fig. 5). No studies have hitherto elucidated the biosynthetic mechanisms underlying the formation of VL in insects. By virtue of the structural similarity between VL and the necic acid moiety of **1**, VL is supposed to be a

cyclization product directly derived from the necic acid of **1**. Given that, it is quite convincing that no trace of VL was formed from **4**, but we cannot determine, at present, which of the two PAs, i.e. lycopsamine and intermedine, served as a precursor for VL. Starting from lycopsamine (2'S,3'S), VL would be readily produced either by the hydrolysis of an ester group, followed by lactonization of the necic acid thereof (viridifloric acid) or simply by intramolecular transesterification. Another possibility remains that intermedine (2'S,3'R) also played a part in VL production. This process, however, entails epimerization at C3' to S form. In fact, this type of steric inversion at C3' [transformation from (+)-trachelanthic acid to (-)-viridifloric acid] has been reported to occur for PAs sequestered by an ithomiid butterfly (Trigo et al., 1994) and an arctiid moth (Trigo et al., 1993). The inability of *E. mulciber* to use compound **2**, of which necic acid is similar in structure to intermedine but methoxylated at C3', may, in part, be due to the lack of enzymes involved in the cleavage or displacement of the ether bond. On the other hand, the formation of VL from **3**, albeit in trace, is very curious, but we have no idea about that. Although the present results do not provide direct evidence for the transformation of the necic acid into VL, it seems tenable that the carbon framework of 2,3-dihydroxy-2-isopropylbutanoic acid that constitutes the necic acid of certain PAs is incorporated into VL.

The finding that male adults derived from the larvae fed with a non-host PA-containing plant (*P. laevigata*) produced a small but

significant quantity of VL (Fig. 5), undoubtedly demonstrates that the larvae are able to assimilate, store, and retain PAs up to adults. Concerning the manipulation of PAs by immatures of butterflies, evidence is already given in many danaiids and ithomiids that larvae still maintain the capacity for assimilating and storing PAs (Rothschild and Edgar, 1978; Trigo and Motta, 1990). The question is what kinds of PAs in *P. laevigata* were available for VL biosynthesis. Four major PAs are known from the plant (e.g. Abe and Yamauchi, 1987; Abe et al., 1991). Of these, 2 PAs could be enumerated as possible candidates for precursors; parsonine, a unique keto-dihydropyrrolizine esterified with viridifloric acid and 17-methylparsonsianidine, a macrocyclic PA with a trachelanthic acid moiety. The same situation would be true of *L. leuconoe* that secretes VL as the major component. In addition, *E. treitschkei aenea* reared on *P. spiralis*, its host plant containing PAs, has been reported to selectively produce and store lycopsamine in the body that is believed to have arisen by selective in vivo hydrolysis of some of the food plant macrocyclic PAs with a viridifloric acid moiety (Edgar, 1982).

4.4. Derivation of ET and the variance of its quantity with age

The data presented in Fig. 6 strongly suggest de novo biosynthesis of ET from nutrients taken up from larval diet. Evidence for this is that the 3 larval food plants proved to contain no traces of either ET or dihydroedulan, and that a nutrient (sugar) acquired as adults exerted no

significant effect on the amount of ET formed. Although one should expect the involvement of other plant secondary metabolites in ET production, it is very unlikely that the 3 food plants of different families share terpenoid precursor(s) with edulan or related skeletons. In fact, de novo biosynthesis of terpenoids is widely known in many insects (Morgan, 2004).

Since day-0 adults had no appreciable quantity of ET, it is apparent that ET biosynthesis starts only after eclosion, progresses further in the imaginal stage, and thereafter declines in some degree with age (Fig. 7). Based on the content of ET stored by male adults, they seem to sexually mature approximately two weeks after eclosion.

4.5. PAs as attractants for male adults

With respect to PA-Lepidoptera interactions, one of the fundamental concerns has been the nature of chemical signal(s) that mediate orientation to PA sources. PAs themselves are thought to barely attract butterflies due to their low volatility. Extensive trapping experiments with various baits including withered plants, PAs, and related compounds and an electrophysiological study have revealed that volatile products emanating from degraded PAs are key substances that enable insects to locate PA sources: Unidentified volatile(s) derived from necic acids are suspected to be a powerful attractant for ithomiid butterflies, while necine base-derived hydroxydanaidal attracts arctiid moths (Pliske et al., 1976; Krasnoff and Dussourd, 1989; Bogner and Boppré, 1989).

However, our results (Fig. 8) demonstrate that certain PAs composed of particular necic acids do serve as attractants at least for *E. mulciber* males. The possibility that as yet unidentified trace breakdown products of PAs worked as attractants cannot absolutely be ruled out. However, hydroxydanaidal and free necic acids as likely attractants were not detected from compound **1**, though it was contaminated with a small amount (less than 5%) of retronecine (**4**). Accordingly, intermedine and/or lycopsamine per se could be regarded as the real olfactory principle to lure the butterfly. Compound **1** also seemed attractive to *P. sita* males, but it appears that in general, a considerable concentration of a PA is required to attract danaids (Honda et al., unpublished).

4.6. Adaptive significance of the linkage between sensory reception and utilization of PAs

Our previous paper has shown that males of *P. sita* only showed a positive feeding response to compound **1** which, among the PAs tested, they can convert most efficiently to danaidone (Honda et al., 2005). This trait seems very adaptive in view of their reproductive strategy. In contrast, despite the very poor availability of **2**, *E. mulciber* males positively responded in feeding to the compound and even to **4** that is apparently useless for VL biosynthesis. The males may perhaps take up such PAs for defensive use. Interestingly enough, however, the olfaction of males is finely tuned to particular PA(s) that are indispensable as precursors for the efficient biosynthesis of VL.

Therefore, they could successfully find and acquire necessary PA(s) in the field. The close relationship with and strong requirement for intermedine/lycopsamine were unusually prominent in *P. sita*. The same appears to hold true of *E. mulciber*.

Obviously males of *E. mulciber* produce androconial chemicals of two different biosynthetic origins: One (VL) is highly likely to be derived from particular PAs with viridifloric or trachelanthic acid moiety which males acquire as adults from plants, and the other (ET), from nutrients taken over from larvae by way of de novo biosynthesis (Fig. 9). Although nothing is known of the roles of ET and VL in the sexual communication of *E. mulciber*, it is feasible that these elaborate and predominant components act as the sex pheromones.

The details of the stereochemical pathway of VL biosynthesis and the biological function of ET and VL are under investigation.

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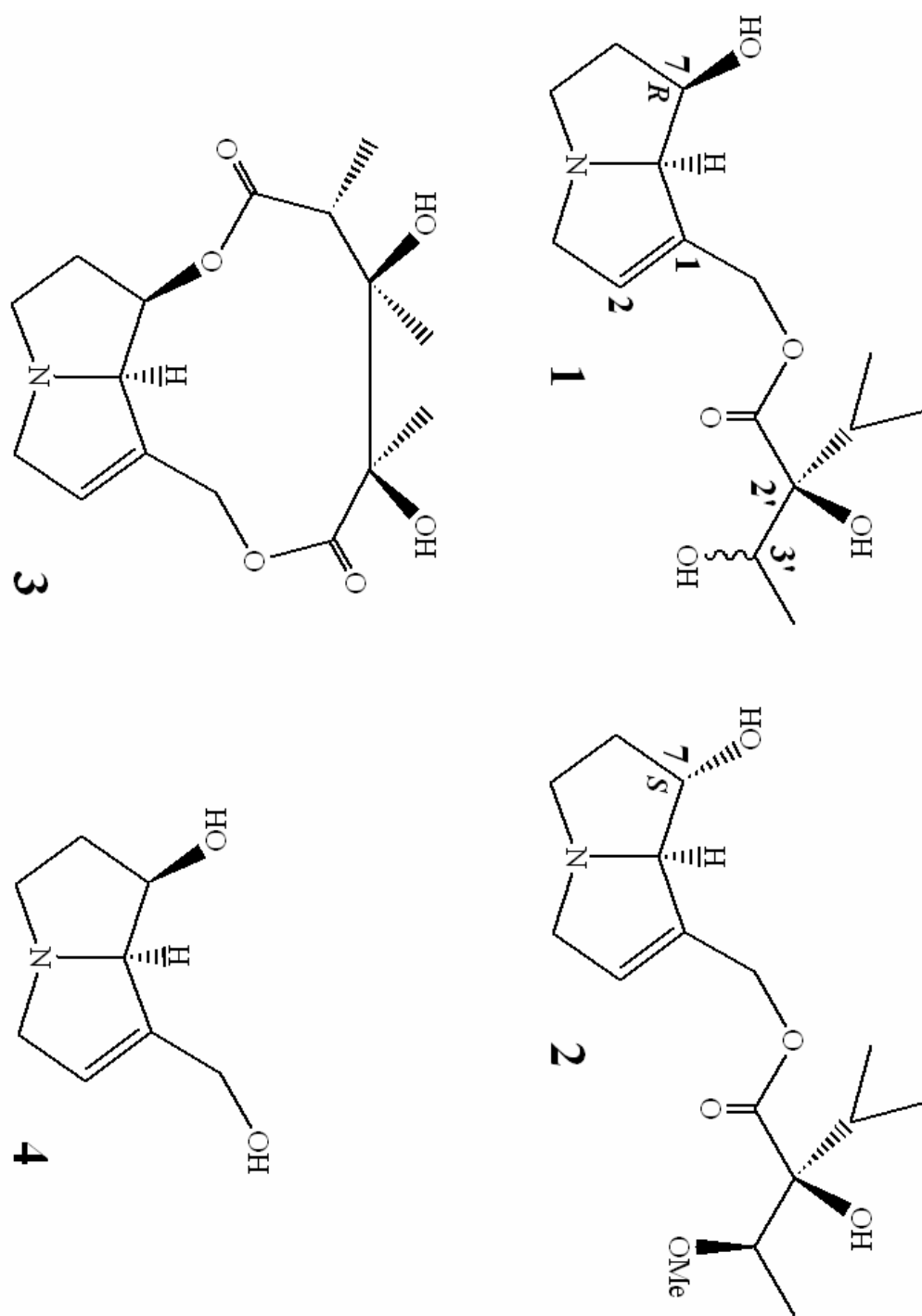
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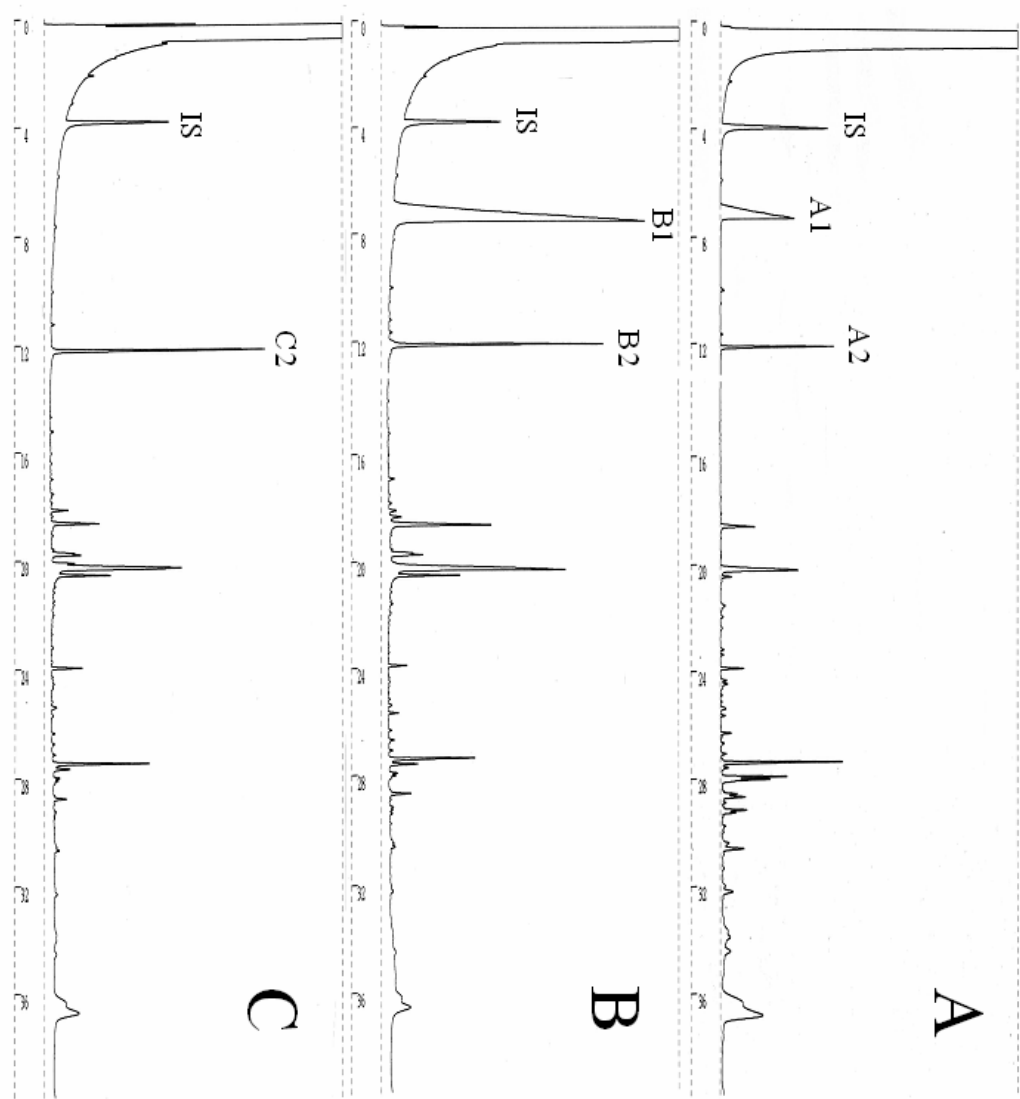
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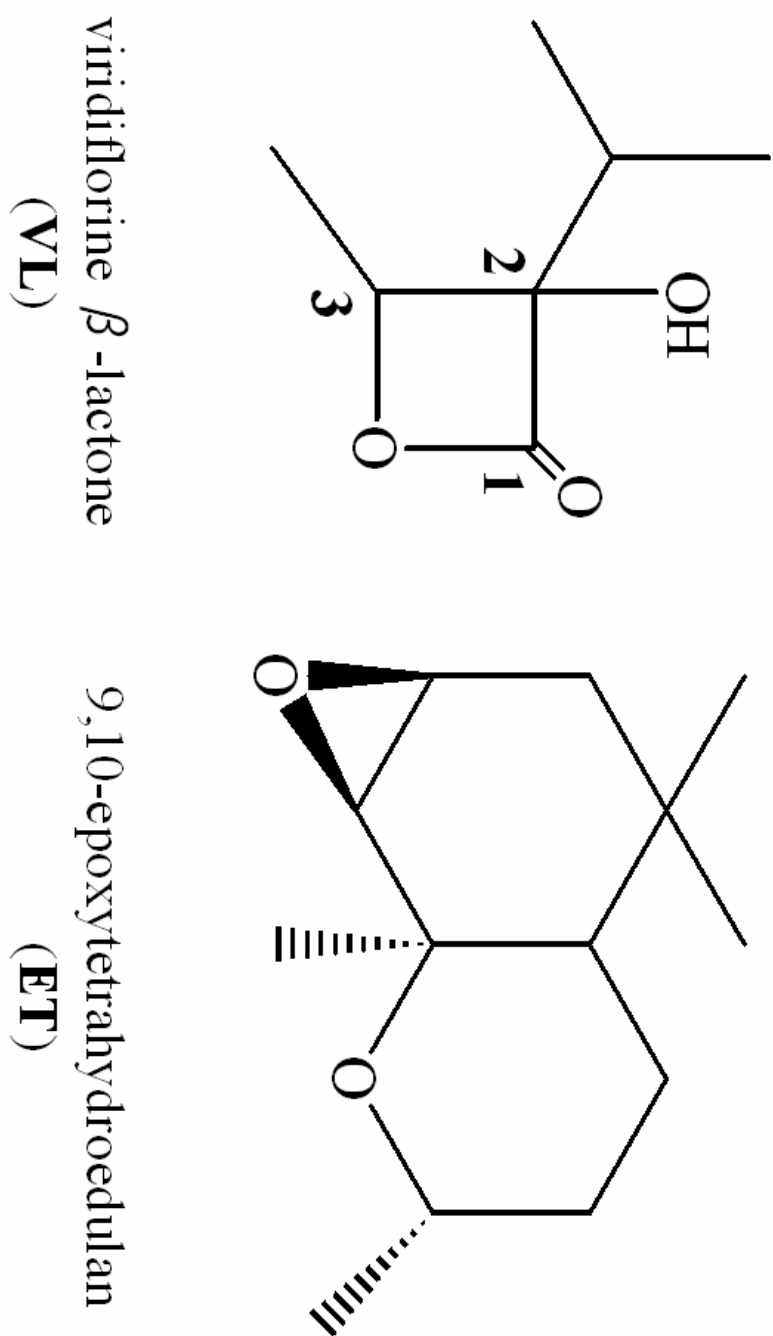
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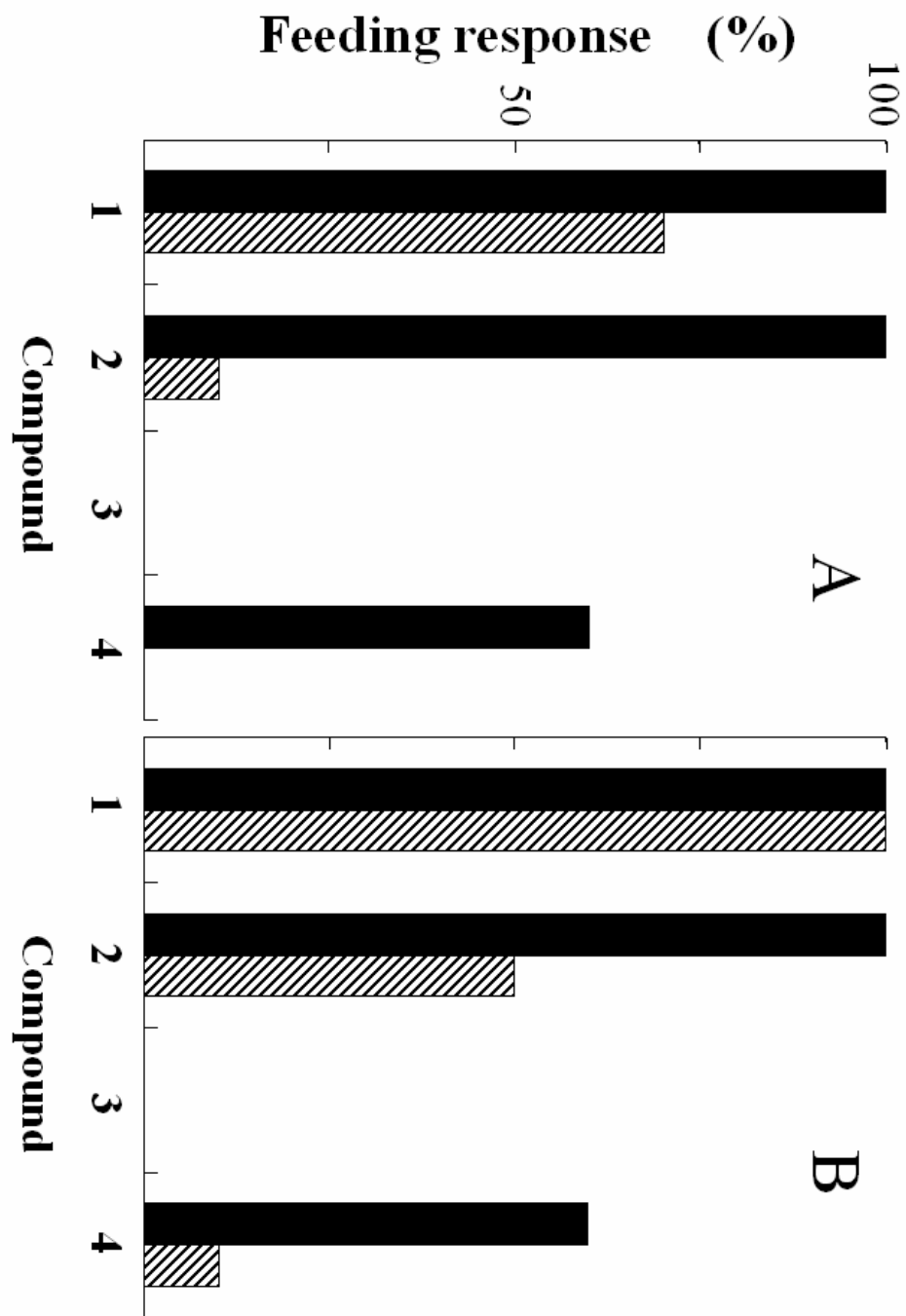
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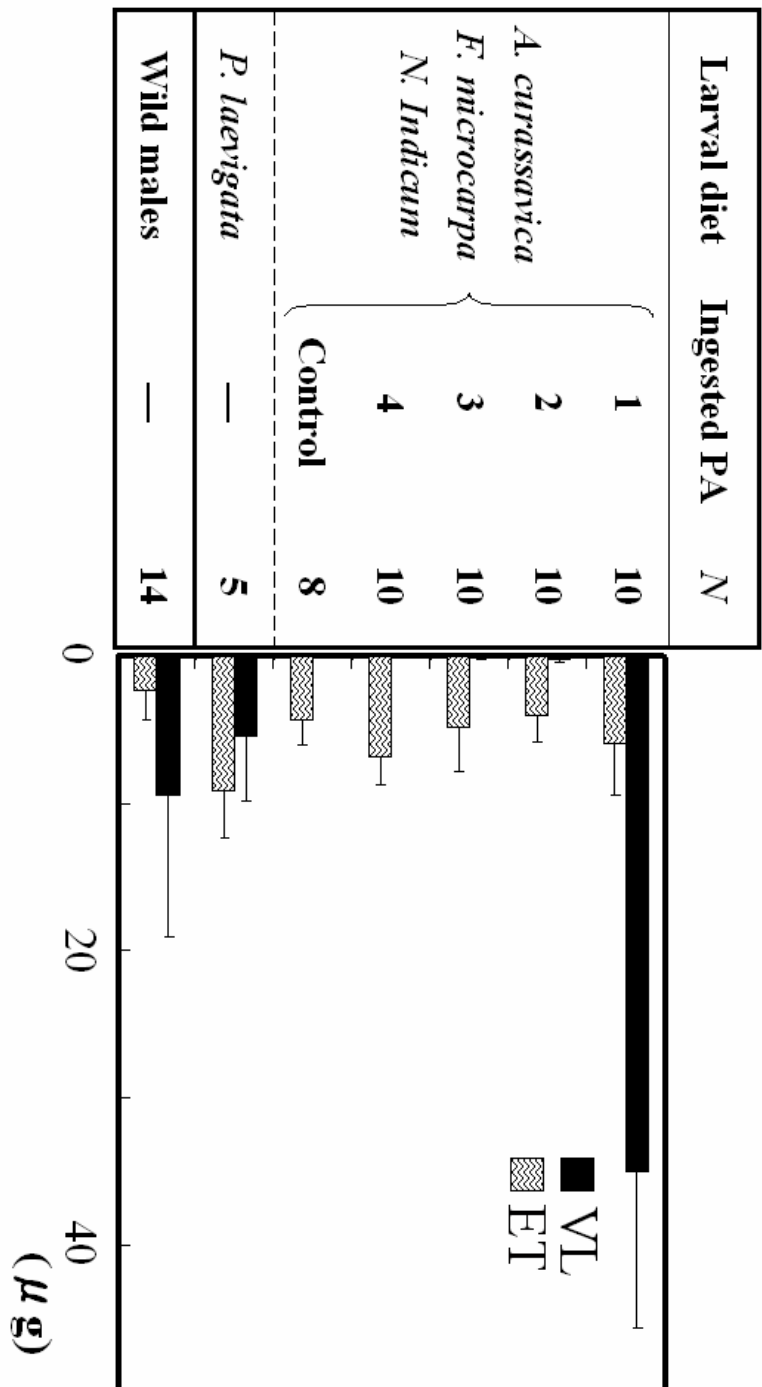
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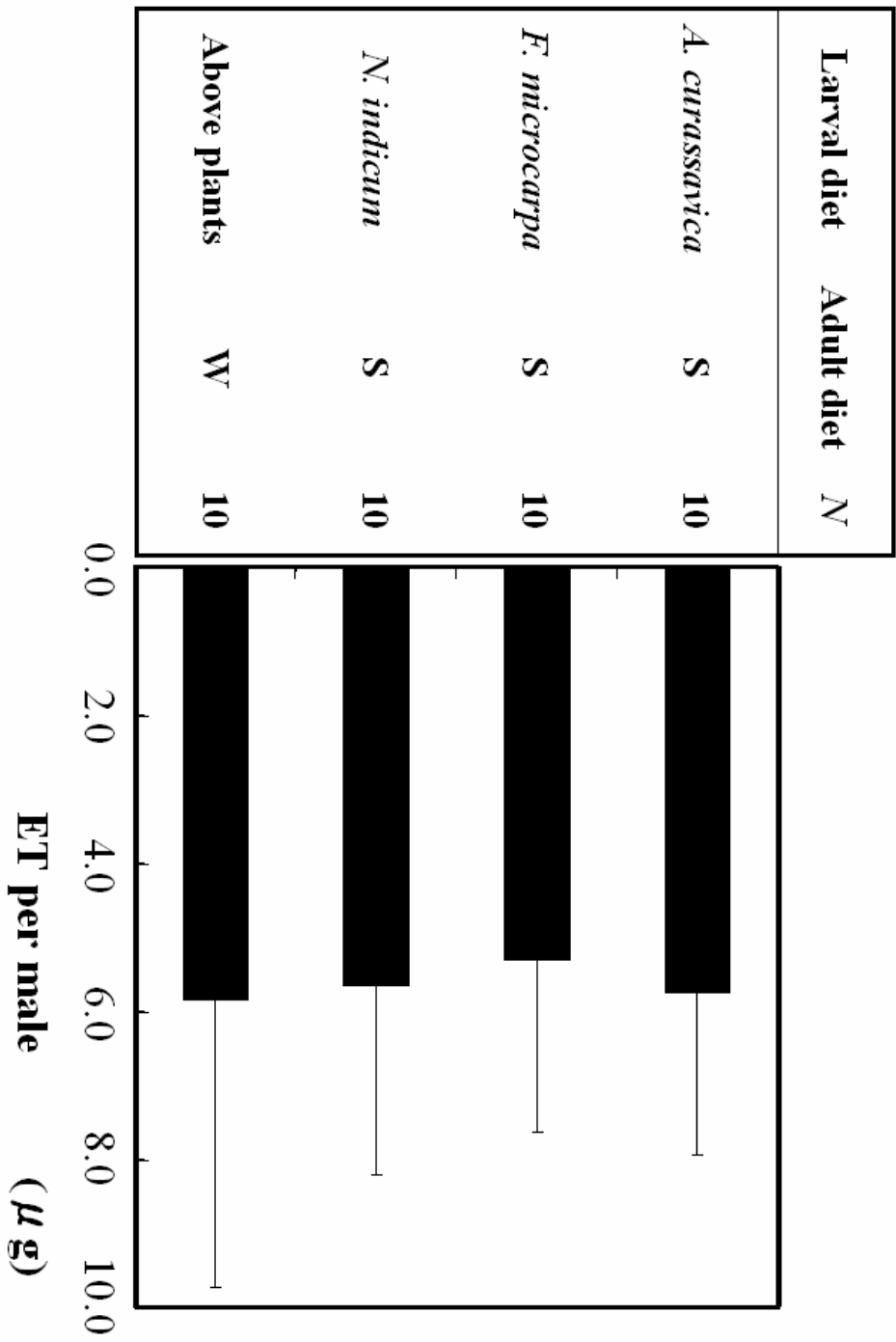
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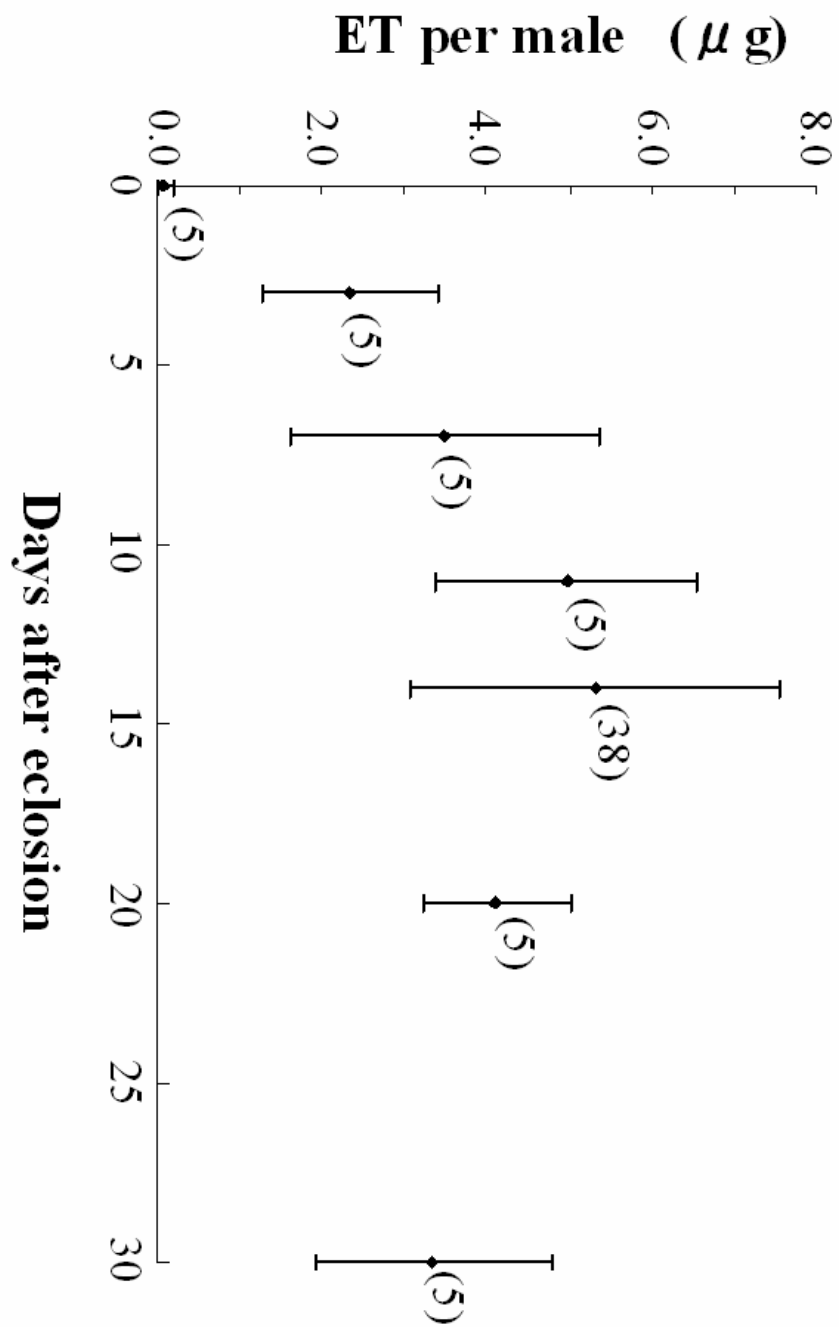
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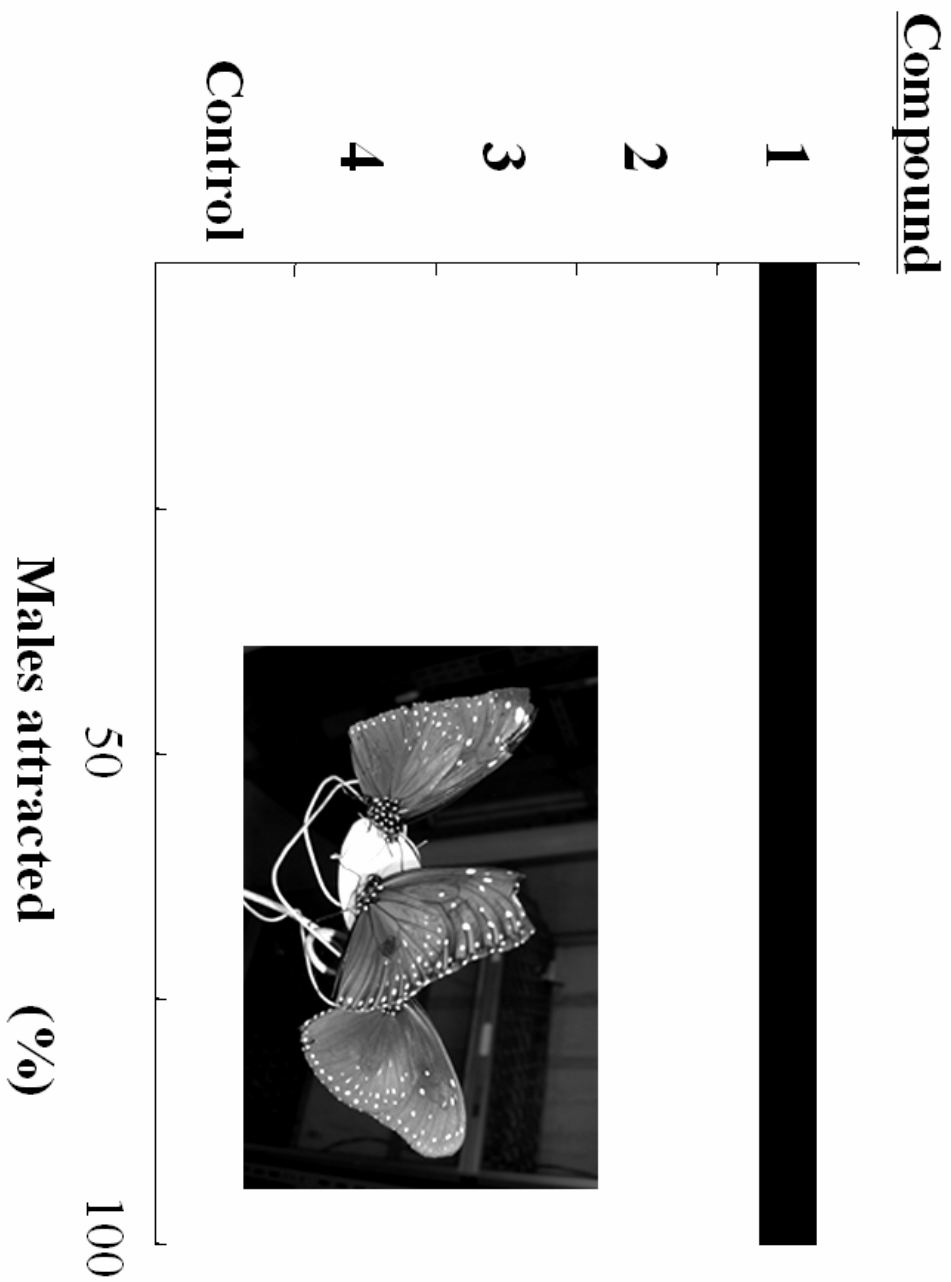
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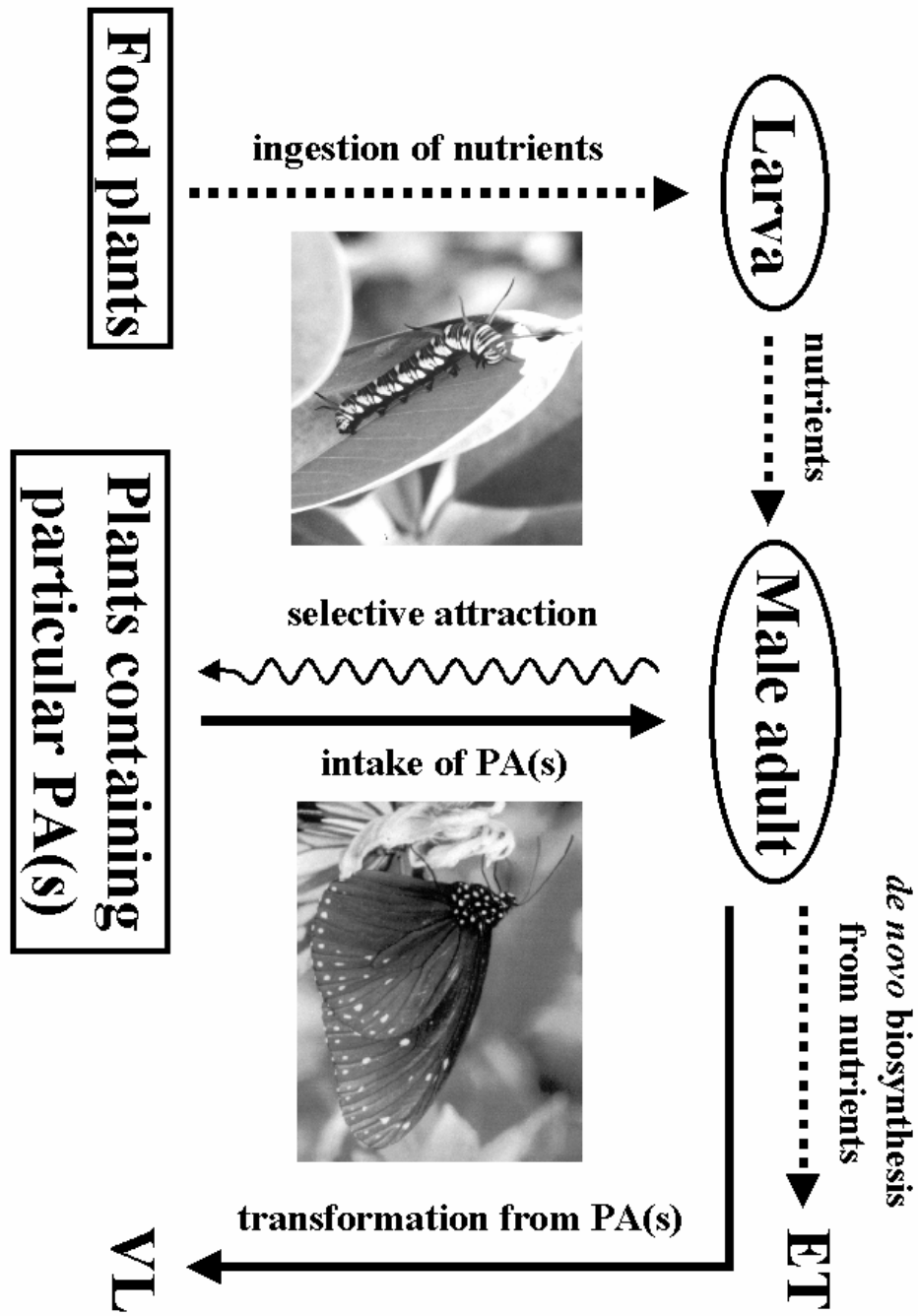
(Fig. 6)



(Fig. 7)



(Fig. 8)



(Fig. 9)

(Legends to Figures)

Fig.1. Chemical structures of pyrrolizidine alkaloids fed to *E. mulciber* males. 1: intermedine (3'R)/lycopsamine (3'S), 2: heliotrine, 3: monocrotaline, 4: retronecine.

Fig.2. Representative GC chromatograms of the hairpencil extracts of *E. mulciber* males. A: field-caught male; B: male that ingested compound **1** (see Fig.1); C: control (PA-unfed) male. IS: internal standard (benzyl alcohol); Peaks A1 and B1: viridiflorine β -lactone; Peaks A2, B2, and C2: 9,10-epoxytetrahydroedulan.

Fig.3. Major volatile components identified from the hairpencils of *E. mulciber* males. VL is a (2S,3S)-form or its enantiomer.

Fig.4. Feeding response to PAs. A: 7-day-old males (N=10). B: 26- to 40-day-old males (N=10). Compound numbers correspond to those of PAs shown in Fig. 1. For compound **3**, its HCl salt (monocrotaline hydrochloride) was used in this test. Black bar: individuals that actually fed on PA solutions, Gray bar: individuals that spontaneously extended the proboscis upon tarsal contact with PAs.

Fig.5. Amounts of VL and ET (mean + SD) in the hairpencils of *E. mulciber* males subjected to PA-administration tests or captured in the

field. PA numbers correspond to those of PAs shown in Fig. 1. For compound **3**, its HCl salt was used. Individual males except wild ones were derived from larvae reared on normal host plants (PA-lacking), i.e. *A. curassavica*, *F. microcarpa*, or *N. indicum* or on a PA-containing non-host, *P. laevigata*. Indoor males were fed daily with 15% aq. sucrose after eclosion.

Fig.6. Effect of larval and adult diets on the production of ET (mean + SD) in the hairpencils of *E. mulciber* males. Fourteen-day-old individuals that were fed daily with 15% aq. sucrose (S) or water alone (W) after eclosion. Each individual fed with water alone originated from larvae reared on any one of the 3 food plants given above. The result suggests that neither secondary metabolites present in the larval food plants nor adult diets are responsible for ET formation.

Fig.7. Age dependence of ET formation (mean \pm SD).

The number of individuals examined is given in parentheses. For the value of day 14, data presented in Figs. 5 and 6 are collectively shown.

Fig.8. Attraction test of *E. mulciber* males to PAs.

A total of 10 males were used. Compound numbers correspond to those of PAs shown in Fig. 1. The photograph shows males gathering and sucking on a paper disc treated with intermedine/lycopsamine (**1**).

Fig.9. Proposed biosynthetic process of ET and VL in male adults of *E. mulciber*.