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## THE "RAISON D'ÊTRE" OF PYRROLIZIDINE ALKALOIDS IN *Cynoglossum officinale*: DETERRENT EFFECTS AGAINST GENERALIST HERBIVORES

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**Abstract**—In this study we tested whether pyrrolizidine alkaloids (PAs) of *Cynoglossum officinale* serve as antifeedants against herbivores. Total PA N-oxide extracts of the leaves significantly deterred feeding by generalist herbivores. Specialist herbivores did not discriminate between food with high and low PA levels. Three PAs from *C. officinale*, heliosupine, echinatine, and 3'-acetylchinate, equally deterred feeding by the polyphagous larvae of *Spodoptera exigua*. Although the plants mainly contain PAs in their N-oxide form, reduced PAs deterred feeding by *S. exigua* more efficiently than PA N-oxides. On rosette plants, the monophagous weevil *Mogulones cruciger* significantly consumed more of the youngest leaves, which had the highest PA level and the highest nitrogen percentage. Larvae of *Ethmia bipunctella*, which are oligophagous within the Boraginaceae, did not discriminate between leaves. All generalist herbivores tested significantly avoided the youngest leaves with the highest PA levels. In the field, the oldest leaves also were relatively more damaged by herbivores than the youngest leaves. It is hypothesized that the skewed distribution of PAs over the leaves of rosette plants reflects optimal defense distribution within the plant.

**Key Words**—*Cynoglossum officinale*, Boraginaceae, pyrrolizidine alkaloids, chemical defense, specialist herbivores, generalist herbivores, *Ethmia bipunctella*, *Mogulones (Ceutorhynchus) cruciger*, *Spodoptera exigua*, *Helix aspersa*, *Frankliniella occidentalis*, *Locusta migratoria*, *Lyriomyza trifolii*.

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## INTRODUCTION

Like philosophers who search for the meaning of life, chemical ecologists look for the "raison d'être" of secondary plant substances (Fraenkel, 1959) or, more specifically, alkaloids (Wink, 1993). Pyrrolizidine alkaloids (PAs) are mainly found in the Asteraceae and the Boraginaceae (Hartmann, 1991). Although the toxicity of PAs to vertebrates has been well documented (Greimer, 1900; Mattocks, 1986; Anonymous, 1989; Wink, 1993), their role as defenses against insect herbivores is less clear. It is often assumed "intuitively" (Wink, 1993) that PAs protect the plant against insect herbivores as well, although evidence for this assumption is sparse (Hartmann, 1991) and even contradictory (see Bentley et al., 1984).

Plants containing PAs still can suffer considerable herbivore damage. In the first place, the plants can be damaged by specialist herbivores that have adapted to PAs (Prins and Nell, 1990; Van der Meijden et al., 1991). Secondly, the total PA content may not be related to herbivory, because the different types of PAs in the plant are not equally deterrent (Bentley et al., 1984; Dreyer et al., 1985; Speiser et al., 1992). Thirdly, some plant parts contain less PAs than others (Hartmann and Zimmer, 1986; Hartmann et al., 1989; Van Dam et al., 1994).

In order to generate a hypothesis on the role of PAs in the feeding behavior of herbivores on the whole plant, we have to consider both the plant's and the herbivore's point of view. Young leaves are more valuable for the plant than old leaves, because they have the highest photosynthetic rates and the longest life expectancy (Harper, 1989). Meanwhile, young leaves are more attractive for herbivores (Hodkinson and Hughes, 1982; Soldaat and van der Meijden, 1990) because they contain higher nitrogen and water percentages (Mooney and Gulmon, 1982). According to optimal plant defense theory, the young leaves should thus be protected best (Zangerl and Bazzaz, 1992). Based on theories of coevolution of plants and herbivores (Ehrlich and Raven, 1964), it can be expected that specialist herbivores will not be deterred by high chemical defense levels in the youngest leaves, while generalists will (Cates, 1980).

In this paper we test whether PAs of rosette plants of *Cynoglossum officinale* L. (Boraginaceae) act as defenses against herbivores. PA levels differ significantly between leaves on the same rosette plant; the youngest leaves have 50–190 times higher PA concentrations than the oldest leaves (Van Dam et al., 1994). *C. officinale* contains several PAs, such as echinatine, 3'-acetylechinatine, heliosupine, trachelanthamine, and viridiflorine (De Jong et al., 1990; Hartmann and Witte, 1994; Van Dam et al., 1995), which are mainly present in the N-oxide form (Sykulska, 1962; Van Dam et al., 1995).

*C. officinale* is a host plant for a range of herbivores (De Jong et al., 1990). Nevertheless, herbivory on *C. officinale* is usually less than 10% of the leaf

area (Prins and Nell, 1990), indicating that the plants are well defended. For our experiments, we chose two specialist herbivore species, the monophagous weevil *Mogulones cruciger* and the oligophagous larvae of *Ethmia bipunctella*. Both herbivores are commonly found on *C. officinale* in The Netherlands (Prins and Nell, 1990; Prins et al., 1992). We also tested the deterrence of PAs on five herbivore species that are not specialized on *C. officinale*. The polyphagous snail, *Helix aspersa*, is the only one of these five species that is naturally occurring in the plant population we studied (Gittenberger et al., 1970). *Lyriomyza trifolii*, *Frankliniella occidentalis*, and *Spodoptera exigua* all are imported, horticultural pest species from different orders within the Insecta. Finally, we used *Locusta migratoria*, which is an oligophagous leaf-chewer that mainly feeds on grasses (Bernays and Chapman, 1977).

#### METHODS AND MATERIALS

*Plant Species.* *Cynoglossum officinale* L. (Boraginaceae) is a facultative biennial that is commonly found throughout the temperate zones of Europe and Asia (De Jong et al., 1990). The plants used in these experiments originated from the calcareous dune area, Meijendel, near The Hague, The Netherlands. In addition to PAs, the leaves of *C. officinale* are known to contain several other secondary metabolites, such as flavonoids, naphthoquinones, and saponins (Hegnauer, 1964; De Jong et al., 1990).

*Herbivores.* *Mogulones (Ceutorhynchus) cruciger* Herbst (Coleoptera, Curculionidae) is monophagous on *C. officinale* (Freese, 1990). The genus *Ceutorhynchus* has recently been revised by Colonelli (1986). The name of this weevil, therefore, has been changed to *Mogulones cruciger* (Heijerman, 1993). The adults feed on the leaves, and the larvae develop in the rootcrown (Dieckmann, 1972), by which they significantly reduce plant weight and seed output (Prins et al., 1992). Adults of *M. cruciger* were sampled in the coastal dune area, Meijendel, The Netherlands, and were kept on leaves of *C. officinale* until they were used for experiments. The larvae of *Ethmia bipunctella* F. (Lepidoptera, Ethmiidae) are oligophagous and feed exclusively on leaves of Boraginaceae (Sattler, 1967). Larvae of *E. bipunctella* were also sampled in Meijendel and propagated on *C. officinale* leaves in the lab (25°C/16 hr light, 18°C/8 hr dark, 70% relative humidity). For the experiments we used second and third stage lab-reared larvae.

*Helix aspersa* Müller (Gastropoda, Helicidae) occasionally damages leaves of *C. officinale* plants in our experimental garden (K. Vrieling, personal communication). *H. aspersa* individuals were sampled in the garden around the laboratory and kept on wet filter paper and nettle leaves at room temperature before they were used in the experiments.

*Lyriomyza trifolii* Burgess (Diptera, Agromyzidae) is a polyphagous leaf-miner, known to feed on over 100 plant species (Harborne, 1988). The larvae feed in the leaf on the mesophyll layer, producing contorted mines (Minkenberg and van Lenteren, 1986). *Frankliniella occidentalis* Pergande (Thysanoptera, Thripidae) also is an extremely polyphagous herbivore. Both adults and larvae feed by piercing leaf cells with their mandibles and ingesting the content through the stylet (Hunter and Ullman, 1989). *Frankliniella* spp. are deterred by lupine alkaloids (Gustafsson and Gadd, 1965). Both *L. trifolii* and *F. occidentalis* were reared on chrysanthemum plants, cv. Ultra Light (20°C continuous 12 L:12 D photoperiod, 70% relative humidity, and 25°C continuous 16 L:8 D photoperiod, 70% relative humidity, respectively). The larvae of *Spodoptera exigua* Hübner (Lepidoptera, Noctuidae) are polyphagous leaf-chewing herbivores (Heath, 1983). *S. exigua* and other *Spodoptera* species are commonly used in antifeedant tests and are known to be sensitive to several types of alkaloids (Aerts et al., 1992; Krug and Proksch, 1993). The larvae were reared in our laboratories on an artificial diet (Aerts et al., 1992) at 25°C/16 hr light, 18°C/8 hr dark and 70% relative humidity. For the experiments we used second to fourth stage larvae. Adults of *Locusta migratoria* L. (Orthoptera, Acrididae) were kept on grass at room temperature before experimentation.

**Choice Tests.** Disks (2 cm diam.) were punched from young, fully expanded leaves and from old leaves of rosette plants of *C. officinale*. Two disks were placed in every Petri dish (9 cm diam.), which was lined with wet filter paper. One herbivore was placed in each Petri dish. All choice tests were replicated 10 to 20 times, depending on availability of the species. The choice tests with *L. migratoria* were conducted in transparent plastic cups, 9 cm diam., 7 cm high. The disks were fixed in the cups with needles.

We tested two choice situations: a disk of a young leaf versus a disk of an old leaf, and a disk of an old leaf treated with MeOH (control) versus a disk of an old leaf treated with PA N-oxide solution in MeOH. Every disk was painted with 3 µg PA/mg fresh weight (FW), which is comparable to the mean PA level of the youngest three to four leaves. The control disks were treated with the same volume of pure MeOH to rule out solvent effects. The area eaten was scored after three days, except for the *L. migratoria* adults, which were so voracious that the amount eaten had to be scored after one day.

**Deterrence of Different PAs.** The difference in deterrence by different types of PAs was tested with *S. exigua* larvae. For this experiment leaves of lettuce (*Lactuca sativa* L.) were used as a carrier for the PA solutions, since they are well accepted by *S. exigua* larvae and do not contain PAs. The deterrence of the PAs was tested in a no-choice situation, using lettuce disks (2 cm diam.) treated with MeOH or a methanolic solution of either heliosupine, 3'-acetylochinate, or echinate. Since we did not want to fully deter feeding, we applied a relatively low dose of 0.5 µg PA/mg FW. Dose-response experiments with total PA extracts showed that at this concentration the feeding

deterency was about 50% relative to control disks (Van Dam et al., unpublished data). Four disks treated with the same solution were placed in a Petri dish lined with wet filter paper. Per Petri dish, two second or third stage *S. exigua* larvae were allowed to feed overnight. Every treatment was replicated 10 times. Leaf disks were copied onto quadrille paper and damage was scored as square millimeters area eaten.

*PA N-Oxides versus Reduced PAs.* Disks from lettuce leaves received 2  $\mu\text{g}$  PA N-oxide/mg FW or 2  $\mu\text{g}$  reduced PA/mg FW, respectively. The two treatments were offered in a choice situation to second stage *S. exigua* larva (20 replicates).

*Whole Plant Experiments: Herbivory in the Lab.* Seeds of *Cynoglossum officinale* plants were sampled in Meijndel in 1992, scarified, and soaked for two weeks at 20°C/16 hr light, 10°C/8 hr dark. The seedlings were potted in 1-liter pots, each containing a mix of dune sand and compost (1:1). Nutrients were supplied by adding 3 g Osmocote Plus (15% N, 11% P, 13% K, 2% MgO, Sierra Chem. Co., Milpitas, CA, USA) per pot. At the start of every experiment, plants of the same half-sib family that had equal numbers of leaves were selected and placed in transparent cylinders. The herbivores were released on the ground near the plant. Since the herbivores differed in voracity, the number of individuals released per plant differed. Per plant, either three adults of *M. cruciger* (six replicates), one individual of *E. bipunctella* (10 replicates), two individuals of *H. aspersa* (21 replicates), 20 females of *F. occidentalis* (12 replicates), or one larva of *S. exigua* (20 replicates) were released near every plant. The herbivores were allowed to feed until a substantial amount of damage was visible. After this period, the absolute amount of damage (area eaten) and the leaf area were scored separately for every leaf. For *F. occidentalis* the percentage of leaf area with silver damage was measured.

For the leaf-miner, eight plants were left for 1 hr in the *L. trifolii* breeding room to allow female flies to lay eggs in the leaves. Seven days later the number of large mines was counted. With this method, the combined effect of antixenotic effects on the choice of female flies and antibiotic effects on the larvae was measured.

Some control plants were kept apart to measure PA content, percentage of nitrogen, leaf toughness, and the number of hairs on individual leaves.

*Herbivore Damage in the Field.* On June 23, 1994, ten field populations of *C. officinale* in Meijndel were selected to measure the natural herbivory per leaf on rosette plants. Five populations were in a shaded habitat, and five were in an open habitat. In each population the percentage of area damaged per leaf of 12 randomly selected rosettes was estimated. The presence of *E. bipunctella* and *M. cruciger* was noted. The presence of blotch mines caused by *Agromyza* spp. (De Jong et al., 1990) was scored.

*Extraction and Purification PA N-Oxides.* In order to obtain purified PA N-oxides, dried leaves of *C. officinale* were ground in a Waring blender and

extracted overnight with 10 ml MeOH/g leaf powder. The extract was filtered over cotton wool, and the residue was reextracted twice for 1 hr. The volume of the combined extracts ( $\pm 500$  ml) was reduced in vacuo to  $\pm 15$  ml and was placed on a Silica-60 (Merck) column. The column was eluted with 150 ml dichloromethane (DCM), followed by 200 ml 10% MeOH in DCM, and three times 200 ml MeOH. The PA N-oxides were eluted in the MeOH fraction. Purity and presence of PA N-oxides were checked by TLC on Silica gel 60 pre-coated plates that were developed in DCM-MeOH-NH<sub>3</sub> (82:15:3). PA N-oxides were detected with acetic anhydride and Ehrlich reagent (Molyneux and Roitman, 1980). If the extract was still impure, the extract was placed on a preparative Silica plate (Merck, layer thickness 2 mm), which was developed as above. The PA-containing bands were scratched off, and the silica was extracted three times with MeOH with continuous stirring. The MeOH fraction containing the PA N-oxides was evaporated and the residue dissolved in 25.0 ml MeOH. The concentration of the PA N-oxide solution was determined with a spectrophotometric color reaction (Mattocks, 1967). By omitting the oxidation step in this color reaction, we could specifically determine that the solution contained N-oxides only.

*Extraction and Purification of Individual PAs.* Dried leaf material was extracted with 0.5 M H<sub>2</sub>SO<sub>4</sub>. After 1 hr, Zn dust was added to reduce the PA N-oxides. The acid extract was filtered and extracted twice with DCM to remove apolar impurities. Subsequently the extract was made alkaline with ammonia (25%) and placed on an Extrelut (Merck) column. After 20 min, the column was washed with two times 100 ml DCM. The volume of combined DCM extracts was reduced with a rotary evaporator to  $\pm 10$  ml and placed on a Silica-60 column. The column was eluted with a gradient of DCM-MeOH-NH<sub>3</sub> (60:40:1), DCM-MeOH (1:1), DCM-MeOH (25:75), and pure MeOH. Presence of PAs in the fractions was checked on TLC (system as above). The PA-containing fractions were evaporated, dissolved in MeOH, and the PAs were identified with GC-MS (DB-1 column, method II as described by Witte et al. (1993). The concentration of the solutions containing the PAs was determined with a spectrophotometric color reaction (Mattocks, 1967).

*PA and Nitrogen Analysis.* Individual leaves of five control plants were extracted for PAs as described in Van Dam et al. (1993). Monocrotaline (Sigma, 99% pure) was used as a reference compound in the spectrophotometric color reaction (Mattocks, 1967). The percentage of organic nitrogen in the leaves of two control plants was determined according to the semimicro-Kjeldahl method (Chapman and Pratt, 1961).

*Physical Characteristics.* Leaves of three control rosette plants were sampled separately and weighed to determine FW. After two days of drying at 50°C, the dry weight (DW) was determined in order to calculate the water content. *C. officinale* leaves are covered with thick-walled, unicellular hairs (De

Jong et al., 1990), which might serve as a barrier against small herbivores. Of three other control plants, the number of hairs was counted in two randomly chosen circles of 1 mm<sup>2</sup> per leaf.

Leaf toughness (in Newtons) was measured on the same leaves as the hairiness, with the use of a penetrometer (Prins and Laan, 1988). Every leaf was punctured on four places, avoiding the midvein, in order to obtain an average toughness over the whole leaf.

*Statistical Analysis.* Standard statistical tests were performed with Statgraphics 5.0 (Statgraphics Company, 1992). The differences in physical characteristics and damage between leaves were analyzed with the Friedman analysis of variance, since the groups (leaves) were not independent (Sokal and Rohlf, 1981)

## RESULTS

### Choice Tests

Leaf disks from young and old leaves differed significantly in both PA content and nitrogen percentage; the young leaves had higher levels of both (Table 1). As predicted, PAs did not deter the specialist herbivores *M. cruciger* and *E. bipunctella*. In fact, they ate more of the disks punched from young leaves, but their preference was not significant (Table 1). Both generalists, *S.*

TABLE 1. CHOICE TEST WITH DISKS OF OLD AND YOUNG LEAVES OF *Cynoglossum officinale* AND DISKS TREATED WITH METHANOL (CONTROL) AND PA N-OXIDE (PAs) EXTRACT<sup>a</sup>

	First experiment			Second experiment		
	Old	Young	<i>P</i>	Control	PAs	<i>P</i>
PA (mg/g FW) (SE)	0.93 (0.22)	4.54 (1.36)	0.03	0.93	3.93	
Nitrogen, % (DW) (SE)	3.30 (0.26)	4.30 (0.26)	0.01			
Specialist herbivores						
<i>Mogulones cruciger</i>	4	6	0.48	9	3	0.11
<i>Ethmia bipunctella</i>	4	9	0.33	12	8	0.18
Generalist herbivores						
<i>Spodoptera exigua</i>	19	0	0.0001	19	0	0.0001
<i>Locusta migratoria</i>	16	2	0.002	10	0	0.004

<sup>a</sup>The table gives the number of individuals that chose to eat more of the treatment. *P* values of Wilcoxon's signed-ranks matched-pairs test. SE = standard error of mean.



*exigua* larvae and *L. migratoria* adults, preferred the disks with the lowest level of PAs. In the first experiment they preferred to feed on disks from old leaves, which also had the lowest nitrogen percentage (Table 1). In the second experiment, the disks treated with PA N-oxides were totally avoided by both herbivores. This indicates that the PA N-oxides extracted from *C. officinale* had a strong deterrent effect on these herbivores, which normally do not feed on this plant species.

### Different PAs

Since *S. exigua* larvae were clearly deterred by PAs, they were used as test animals to assess the difference in detergency between individual PAs. After separation and purification of a tertiary PA extract, we obtained three different types of PAs in quantities large enough to perform the detergency test: echinatine [RI = 2172,  $M^+$  ( $m/z$ ) = 299] 88% pure, 3'-acetylechinatine [RI = 2220,  $M^+$  ( $m/z$ ) = 341] 89% pure, and heliosupine [RI = 2557,  $M^+$  ( $m/z$ ) = 397] 87% pure. The purity and identity of the alkaloids were determined with GC-MS. For some unknown reason, we did not obtain saturated PAs such as viridiflorine, although they form 20–30% of the PAs present in the leaves (Van Dam et al., 1995). Although the PA concentration on the leaf disks was relatively low (0.5  $\mu\text{g}/\text{mg}$  FW), all PAs clearly reduced feeding by *S. exigua* larvae compared to the methanol treatment (Figure 1, Kruskal-Wallis analysis of variance,  $H = 13.7$ ,  $P = 0.004$ ). However, there was no statistically significant difference in

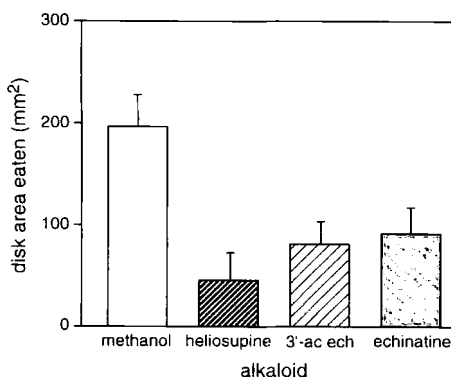


FIG. 1. Feeding detergency of different types of pyrrolizidine alkaloids (PAs) of *Cynoglossum officinale* against *Spodoptera exigua* larvae. All PAs were applied on lettuce disks (0.5  $\mu\text{g}$  PA/mg FW). Abbreviation: 3'-ac ech = 3'-acetylechinatine. Error bars denote standard error of the mean.

deterrency between the single PAs (Kruskal-Wallis on results of PA treatments only,  $H = 3.90$ ,  $P = 0.14$ ).

#### PA N-oxides versus Tertiary PAs

For the reason mentioned above, the difference in deterrency between PA N-oxides and their free bases was tested with *S. exigua* larvae. In a choice test, the larvae clearly preferred disks with PA N-oxides over disks with the same concentration (2  $\mu\text{g}/\text{mg}$  FW) of tertiary PAs (Table 2). However, this does not mean that PA N-oxides are not deterrent at all. In the first choice experiment, both *S. exigua* and *L. migratoria* were deterred by high PA N-oxide concentrations in their food (Table 1).

#### Whole Plant Experiments

*Physical and Chemical Leaf Characteristics.* Again the PA levels differed significantly between the leaves (for results of Friedman analysis of variance see legend to Figure 2): they decreased from the youngest to the oldest leaf (Figure 2a; linear regression on ranks  $R = -0.98$ ,  $P < 0.001$ ). The percentage of nitrogen also declined with leaf age; young leaves had higher nitrogen percentages than old leaves (Figure 2b; linear regression on ranks  $R = -0.98$ ,  $P < 0.001$ ).

Contrary to our expectations, the water content of young leaves was significantly smaller than that of middle-aged leaves (Figure 2c) and was not significantly correlated with leaf number ( $R = 0.54$ ,  $P = 0.21$ ). Not surprisingly, the youngest leaves had more hairs per square millimeter than the older leaves (Figure 2d,  $R = -1.0$ ,  $P < 0.001$ ). Both the increase in water content and the decrease in hairs per area with age probably result from cell stretching. The toughness did not significantly differ between the leaves (Friedman,  $\chi^2 = 7.4$ ,  $P = 0.19$ ) and there was no correlation with leaf number ( $R = 0.01$ ,  $P = 0.98$ ).

TABLE 2. CHOICE TEST WITH GENERALIST HERBIVORE *Spodoptera exigua*<sup>a</sup>

	PA N-ox	tert-PA	P
Mean eaten ( $\text{mm}^2 \pm \text{SE}$ )	42.3 ( $\pm 4.3$ )	13.4 ( $\pm 2.8$ )	0.0004
Animals that ate more of (N)	18	2	

<sup>a</sup>Pyrrrolizidine alkaloid N-oxides (PA N-ox) of *Cynoglossum officinale* and their corresponding free bases (tertiary PAs) were both applied at 2  $\mu\text{g}/\text{mg}$  FW, on disks from lettuce leaves. Differences were analyzed with Wilcoxon's signed-ranks matched-pairs test.

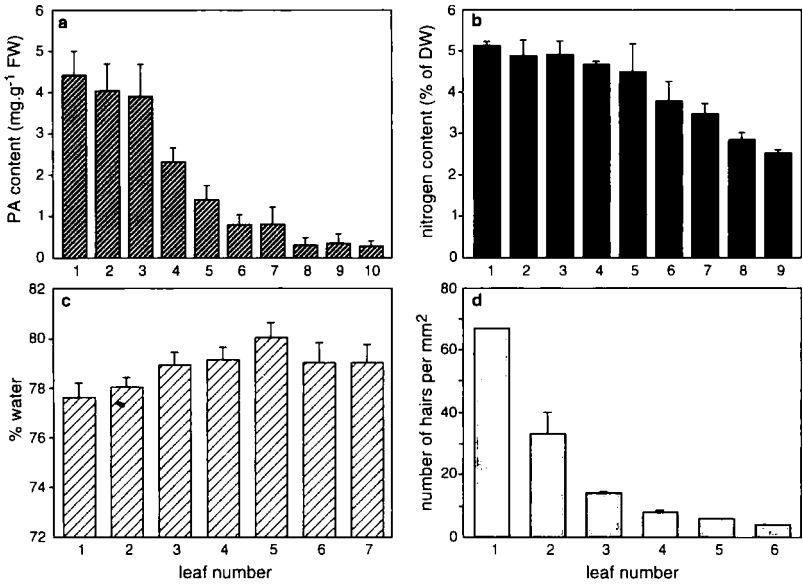


FIG. 2. Chemical and physical leaf characteristics of rosette plants of *Cynoglossum officinale*. Leaf number 1 is the youngest leaf.  $\chi^2$  and  $P$  value of Friedman analysis of variance are given in parentheses after each characteristic: (a) pyrrolizidine alkaloid (PA) level ( $N = 5$ ,  $\chi^2 = 31$ ,  $P = 6.2 \times 10^{-5}$ ); (b) nitrogen percentage ( $N = 2$ ,  $\chi^2 = 14.8$ ,  $P = 0.063$ ); (c) water content ( $N = 8$ ,  $\chi^2 = 13.8$ ,  $P = 0.031$ ); (d) number of hairs ( $N = 3$ ,  $\chi^2 = 15$ ,  $P = 0.01$ ).

**Herbivory in the Lab.** The amount of herbivory was measured as the area eaten (square millimeters) per leaf. We choose to express herbivory as the fraction of the leaf that was eaten (square millimeters per square centimeter or percentage). In this way we corrected for the greater chance that larger leaves have to be found by randomly searching herbivores. Without this correction, the differences in damage between leaves were even greater. The monophagous *M. cruciger* adults significantly preferred young leaves over older leaves (Figure 3a, for results of Friedman analysis see legend to Figure 3; linear regression on ranks,  $R = -0.99$ ,  $P = 0.00001$ ), which may be due to the higher nitrogen levels. PAs again did not deter feeding by these monophagous weevils. *E. bipunctella* larvae did not discriminate between leaves (Figure 3b). The negative correlation between damage and leaf number was not significant ( $R = -0.55$ ,  $P = 0.12$ ). This is consistent with our earlier findings that *E. bipunctella* larvae are not deterred by high PA levels in the younger leaves. However, unlike *M.*

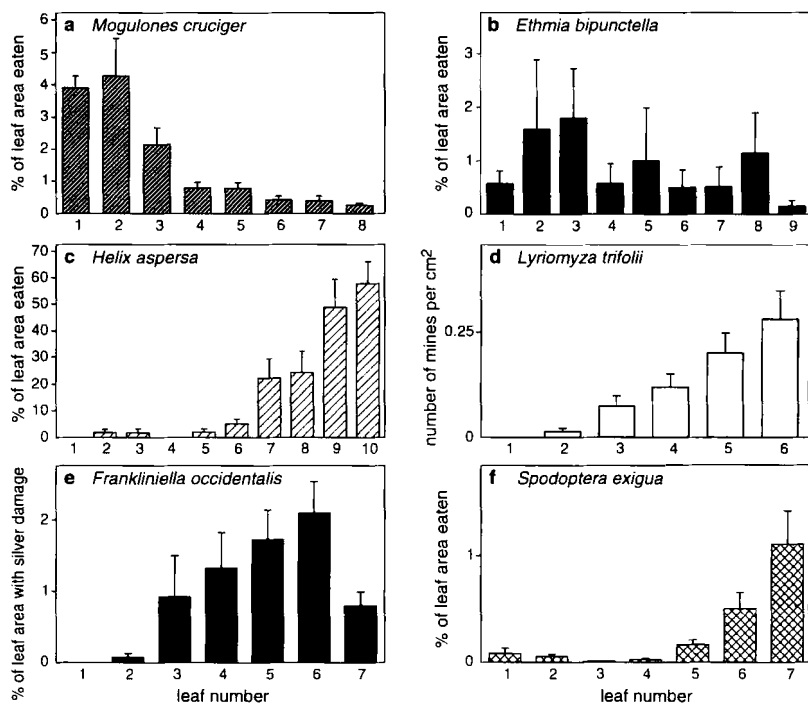


FIG. 3. Fraction of leaves eaten by different herbivores on rosette plants of *Cynoglossum officinale*. Leaf number 1 is the youngest leaf.  $\chi^2$  and  $P$  value of Friedman analysis of variance are given in parentheses after each species name. (a) *Mogulones cruciger* ( $N = 6$ ,  $\chi^2 = 32.7$ ,  $P = 3.10^{-5}$ ); (b) *Ethmia bipunctella* ( $N = 9$ ,  $\chi^2 = 4.66$ ,  $P = 0.79$ ); (c) *Helix aspersa* ( $N = 21$ ,  $\chi^2 = 86.4$ ,  $P = 8.5 \times 10^{-15}$ ); (d) *Lyriomyza trifolii* ( $N = 8$ ,  $\chi^2 = 33.4$ ,  $P = 3.12 \times 10^{-6}$ ); (e) *Frankliniella occidentalis* ( $N = 12$ ,  $\chi^2 = 45.5$ ,  $P = 3.7 \times 10^{-8}$ ); (f) *Spodoptera exigua* ( $N = 19$ ,  $\chi^2 = 40.8$ ,  $P = 3 \times 10^{-7}$ ).

*cruciger*, they did not feed significantly more on leaves with higher nitrogen percentages, as we would have expected.

All generalist herbivores in this experiment significantly discriminated between the leaves (Figure 3c-f). The older the leaves, the more they were consumed (linear regressions on ranks: *H. aspersa*,  $R = 0.92$ ,  $P = 0.0002$ ; *L. trifolii*,  $R = 0.996$ ,  $P = 0.000$ ; *F. occidentalis*,  $R = 0.80$ ,  $P = 0.03$ ; *S. exigua*,  $R = 0.90$ ,  $P = 0.006$ ). Consequently, these generalist herbivores strongly prefer those leaves with a low PA content, which is in accordance with the results of the choice tests (Table 1). Since *L. trifolii* fed and developed in the leaf, the

difference in number of large mines per leaf may be a combination of antixenotic and antibiotic effects of PAs. Because the leaf number differed between experiments, we were unable to correlate herbivory directly to leaf characteristics.

*Herbivory in the Field.* Although in each population at least one specialist herbivore species was present, the youngest leaves overall had relatively less herbivore damage than the older leaves (Figure 4). Since the Friedman analysis requires equal numbers of observations per leaf number, the differences between leaves were analyzed per group of plants with the same number of leaves. Except for the small group of plants with eight leaves, the herbivore damage was significantly different between leaves (Table 3, Friedman analysis). The amount of damage and leaf number were significantly correlated for all groups, which indicated that independent of number of leaves on the plant, the older leaves were relatively more damaged than the youngest leaves.

The average damage on the leaves, 3.8%, was comparable to earlier reports (Prins and Nell, 1990). Mines of *Agromyza* spp. caused a significant loss of photosynthetically active leaf surface. On average the mines caused a loss of 11.7% of the leaf surface, while leaves without mines suffered only 3.12% damage (Wilcoxon test,  $Z = 8.36$ ,  $P < 0.001$ ). Blotch mines were only found on older leaves, on average on leaf number 4. This indicates that, like *L. trifolii*, the naturally occurring leaf-miners avoid the youngest leaves with the highest PA levels.

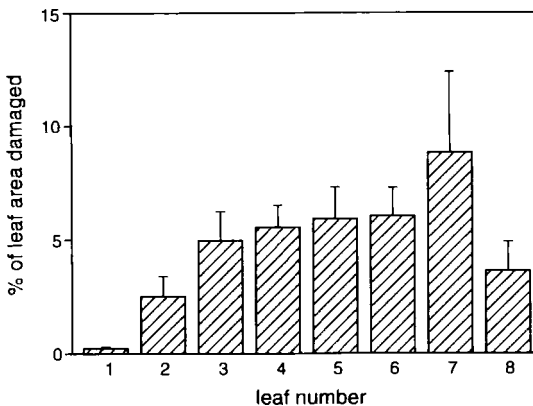


FIG. 4. Average percentage of herbivore damage per leaf, observed on rosette plants of *Cynoglossum officinale* in the dune area in Meijendel near The Hague, The Netherlands, on June 23, 1994. Leaf number 1 is the youngest leaf.

TABLE 3. RESULTS OF FRIEDMAN ANALYSIS OF VARIANCE AND SPEARMAN RANK CORRELATION ANALYSIS FOR DIFFERENCES IN HERBIVORY BETWEEN LEAVES OF FIELD PLANTS

Leaves (N)	Plants (N)	Friedman ANOVA		Spearman RC	
		$\chi^2$	P	R <sup>2</sup>	P
3	17	19.3	$6 \times 10^{-5}$	0.54	0.01
4	41	58.3	$1 \times 10^{-12}$	0.67	<0.001
5	25	53.5	$6 \times 10^{-11}$	0.62	<0.001
6	19	53.0	$3 \times 10^{-10}$	0.71	<0.001
7	9	37.0	$2 \times 10^{-6}$	0.72	<0.001
8	3	10.3	0.17	0.77	<0.001

#### DISCUSSION

In this paper we showed that PAs in *C. officinale* rosettes act as deterrents against generalist herbivores. Despite different ways of feeding, all generalist herbivores avoided the young leaves, which had the highest PA levels. The results of the disk choice tests with PA N-oxide extracts indicate that PAs indeed are responsible for deterring generalist herbivores.

The specialist herbivore species we tested were unaffected by PAs in their food. The monophagous *M. cruciger* preferably consumed the youngest leaves, which are richest in nitrogen, thus maximizing intake of nitrogen. For many insect herbivores, fitness increases with nitrogen intake (Raupp and Denno, 1983; Scriber, 1984). In this case, PAs might even serve as feeding stimulants, since high PA levels coincide with high nitrogen percentages.

In contrast to *M. cruciger*, the oligophagous *E. bipunctella* does not significantly prefer the plant parts with the highest nitrogen levels. However, this has no negative effects on the fitness of *E. bipunctella*. Time to pupation and pupal weight were not significantly different between individuals reared on the oldest three leaves and on the youngest three leaves (Van Dam and Bergshoeff, unpublished data). The difference in nitrogen levels in leaves of *C. officinale* may have been too small to cause significant differences in fitness parameters. Only a factor of 1.6 existed between the youngest three and the oldest three leaves (Figure 2b).

In earlier reports on retarded development of *E. bipunctella* larvae on damaged *C. officinale* plants, it was suggested that this effect on the herbivore's fitness was due to induction of PAs (Prins and Laan, 1988). However, data on

induced changes were lacking. This makes them unsuitable for comparison. Unfortunately, data on the effect of leaf quality on the fitness of *M. cruciger* are hard to get, because these weevils spend their larval stage in the roots.

We found that low concentrations of echinatine, 3'-acetylechinatine, and heliosupine all deterred feeding by a nonadapted herbivore in a no-choice situation. Contrary to our findings, Bentley et al. (1984) reported that echinatine and echinatine N-oxide, in concentrations comparable to those we used, did not significantly deter feeding by spruce budworm. This indicates that not all nonadapted herbivore species are equally sensitive to the antifeedant effect of PAs.

Since all types of PAs we tested reduced feeding equally well, the question arises as to why *C. officinale* maintains such a variety of different PAs. The reason may be that a mixture of compounds is even more effective, because of synergistic effects (Berenbaum, 1985) or because it reduces the probability of adaptation by herbivores (Jones, 1983). Moreover, plants are attacked not only by all sorts of herbivores but also by pathogens. Specific PAs might have evolved as protection against different species of natural enemies. These three hypotheses all need further testing, which is beyond the scope of this paper.

Although the reduced form of PAs seemed more effective as a defense against herbivores (Speiser et al., 1992; this paper), the PAs in the leaves of *C. officinale* almost exclusively occur as N-oxides (Sykulska, 1962; Van Dam et al., 1995). The reason may be that PA N-oxides are better suited for specific phloem transport and safe vacuolar storage within the plant (Hartmann et al., 1989). Moreover, the difference in deterency between the two forms of PAs is only relative. The choice experiment clearly showed that moderate concentrations of PA N-oxides also strongly deter feeding by generalist herbivores.

Furthermore, our experiments showed that the youngest leaves with the highest PA levels suffer less damage from generalist herbivores. Since generalist herbivores form the greatest potential number of enemies (Wink, 1993), we may conclude that, overall, the youngest leaves are better protected against herbivory. However, the fact that these leaves contain the highest PA levels does not preclude that other characteristics may play a role. High hair densities, which coincide with high PA levels in *C. officinale*, may also act as a defense against herbivores.

The damage we observed in the field, of course, is a combination of feeding by specialist and generalist herbivores. Although the damage pattern in the field seems more comparable to that of generalist herbivores, the difference in damage between the leaves is less extreme than in the lab experiments. Nevertheless, these data show that in the field also, the youngest leaves are the best protected. The lab data show that this is not due only to the fact that old leaves have been exposed to herbivory for a longer time. The observation that the youngest leaves are best protected, may reflect the plant's optimal defense strategy. Two aspects

of plant organs are important for optimal defense distribution: the photosynthetic value and the vulnerability of a leaf (Mooney and Gulmon, 1982; Zangerl and Bazzaz, 1992). On both aspects young leaves score higher than old leaves (Harper, 1989). If a rosette plant should have to maintain the same PA level of the youngest leaves throughout the whole plant, the investment in PA synthesis should be more than 50 times higher (see Van Dam et al., 1994). Assuming that it is too costly to do so, investing in defense for young leaves to optimize plant growth thus seems an optimal strategy for *C. officinale* rosettes.

In conclusion, the "raison d'être" of pyrrolizidine alkaloids in rosettes of *C. officinale* is that they protect the most valuable organs of the plant against generalist herbivores. Our paper also shows that to study the possible defensive functions of secondary metabolites, one should test these chemicals against several specialist and generalist herbivores to gain insight into their ecological role.

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