Time-Course of Pyrrolizidine Alkaloid Processing in the Alkaloid Exploiting Arctiid Moth, *Creatonotos transiens*

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The processing of dietary pyrrolizidine alkaloids by larvae and adults of the arctiid moth *Creatonotos transiens* was studied in time-course experiments: In larvae, pyrrolizidine alkaloid uptake is quickly followed by the transformation of the alkaloids into their N-oxides. Furthermore, if 7 *S*-heliotrine is applied, a stereochemical inversion of the hydroxyl group at C 7 to 7 *R*-heliotrine can be observed within 48 h of feeding. The rate of this biotransformation is substantially higher in males which use the 7 *R*-form later as a precursor for the biosynthesis of 7 *R*-hydroxydanaidal, a pheromone. The resorbed pyrrolizidine alkaloids are deposited in the integument within 48 h, where they remain stored during the larval, pupal and partly also the imaginal stages. Virtually no alkaloids are lost during ecdysis. Some pyrrolizidine alkaloids can be recovered from the meconium which is released at eclosion by the imagines especially when disturbed. In the adults pyrrolizidine alkaloids are processed in different ways by the two sexes: In females, about 50–80% of total alkaloids are transferred from the integument to the ovaries and the eggs within 2–3 days after eclosion. If females mate with alkaloid-rich males they additionally receive with the spermatophore up to 290 µg pyrrolizidine alkaloid, which are further translocated to the eggs. A biparental endowment of eggs with acquired defence alkaloids is thus achieved. In males, 30–50% of pyrrolizidine alkaloids remain in the integument; about 10–30% are transferred to the scent organ, the corema, where they are converted into 7 *R*-hydroxydanaidal. Another part (about 40%) is passed to the spermatophore. In the laboratory experiments, the sizes of the coremata and their respective 7 *R*-hydroxydanaidal contents are strongly dependent on the availability of dietary pyrrolizidine alkaloids during L 6 and especially L 7 stages. In the L 7 stage even short-term feeding (4–6 h) on *Senecio jacobaea* is sufficient to induce large coremata.

Introduction

Insect herbivores cope with the plants' defence chemistry in various, often species-specific fashions: 1. Polyphagous species often recognize plants which store noxious allelochemicals and avoid them. 2. Polar compounds are often not resorbed but eliminated with the faeces. 3. If resorption does take place, most herbivores have efficient detoxicating enzymes. 4. Some herbivores are adapted to a specific allelochemical and may even use it as an acquired defence compound. 5. A few species even have more elaborate ways to exploit the plants' defence chemicals as pheromone precursors or morphogens [1–4].

A number of arctiid moths (Lepidoptera) are known to exploit some dietary allelochemicals. Among them are two species of the South Asian genus *Creatonotos* which have an elaborate way to cope with pyrrolizidine alkaloids (PA) (for reviews [5–7]). Pyrrolizidine alkaloids were found to be phagostimulants for larvae of *Creatonotos transiens* [2]. Dietary pyrrolizidine alkaloids are resorbed from the gut lumen with the aid of a specific intestinal carrier system [8]. Pyrrolizidine alkaloids are then transferred to the larval integument where they remain stored through the subsequent life stages [9]. When 7 *S*-heliotrine is applied, a stereochemical inversion of the hydroxyl group at C 7 into 7 *R*-heliotrine takes place [10]. After eclosion, substantial amounts of pyrrolizidine alkaloids are transferred to the ovaries and eggs in females, whereas in males a smaller part is converted into the pheromone, 7 *R*-hydroxydanaidal [11]. In males, pyrrolizidine alkaloids serve still another function (at least in laboratory cultures [11]) as a morphogen which induces the full development of a large abdominal scent organ, the corema [12, 13].

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In this communication we report on the time-course of pyrrolizidine alkaloid processing in larvae and newly hatched imagines of *Creatonotos transiens:* Emphasis was laid on the question of the time dependency of 1. the biotransformation of 7*S*-heliotrine into 7*R*-heliotrine and their N-oxides, 2. pyrrolizidine alkaloid storage in the integument, 3. the transfer of pyrrolizidine alkaloids into the ovaries and eggs in females, 4. the formation of the pheromone 7*R*-hydroxydanaidal in the male corema, 5. the induction of the corema anlage, and 5. the possible endowment of females with pyrrolizidine alkaloids by copulating males.

Materials and Methods

Feeding experiments

A. Larvae. C. transiens has 7 larval stages, termed L 1 to L 7 in the following. A laboratory culture of *Creatonotos transiens* was started with animals from the Philippines as described in [6–11] and maintained continuously in captivity for over 14 months. L 5 to L 7 larvae were kept singly in Petri dishes and reared on a semiartificial diet (without any antibiotics or preservatives), onto which defined amounts of pyrrolizidine alkaloids (7*S*-heliotrine (from Chemasea, Manuf. PTY. Ltd., Sydney, Australia)), senecionine or seneciphylline (kindly provided by R. Molyneux, USDA Albany) were given. For some experiments leaves of *Senecio jacobaea* were supplied alternatively. At the intervals mentioned in the tables and figures animals were harvested, dissected into gut (food residues included), haemolymph, fat body, sex organs, and integument, immediately frozen to −20 °C and stored at this temperature until alkaloid extraction. Faeces were processed accordingly. In parallel experiments we injected defined amounts (up to 30 µl) of aqueous pyrrolizidine alkaloid solutions (adjusted to pH 7) laterally into the larval haemolymph. Larvae were slightly anaesthesized with ether for about 15 sec prior to injection. Before and after the application of pyrrolizidine alkaloids the animals had no period of starvation.

B. Imagines. The time of eclosion was monitored separately for each animal. At the time interval given the moths were collected and processed in a sex-specific way: Females were killed by decapitation and their ovaries were removed. Males were decapitated and their abdomina were subjected to air pressure so that the coremata became fully inflated [13]. After measuring the respective size of the corema, the scent organ was cut off and weighed on an electronic balance. Animals/tissues were stored at −20 °C.

Alkaloid extraction and gas-liquid chromatography

Animals/tissues were ground in a mortar in 10 ml 0.5 M HCl. Zinc powder (about 500 mg) was added to reduce pyrrolizidine alkaloid-N-oxides. The homogenates were left standing overnight at room temperature. Next day they were made alkaline with 6 M NaOH and poured onto Chem elut columns (Analytichem, ICT, Frankfurt) for solid-phase extraction. CH_2Cl_2 was used as an eluent. The eluate was collected and evaporated *in vacuo*. Crude pyrrolizidine alkaloid extracts were taken up in MeOH and analyzed by capillary GLC, using a Varian 3300 instrument which was equipped with a nitrogen-specific detector. Column: DB 1, 30 m × 0.3 mm, film thickness 1 µm. GLC conditions: Oven: 190 °C to 300 °C with 20 °C/min, then 5 min isothermal. Injector: 250 °C, split injection (1:20); detectors: 300 °C; carrier gas: helium 90 kpascal; make up gas: nitrogen. 7*S*-Heliotrine, seneciphylline or senecionine were used as external standards for quantification (Spectra physics integrator SP 4270). Coremata were suspended in 200 µl ethyl acetate; their extracts were analyzed by capillary GLC without further purification. 7*S*-Heliotrine and its metabolites were identified by GLC-MS in previous studies [10–12]; in this study these compounds were identified according to their specific retention indices.

About 1600 insects were treated with alkaloids and later analyzed by capillary GLC for the experiments described in this publication. All values given in tables or figures are means of measurements of 3–14 animals which were processed individually. Pyrrolizidine alkaloid metabolism (*i.e.* uptake, biotransformation, storage and degradation) seems to differ quantitatively between animals. Although the larvae were kept under controlled conditions, we could not totally eliminate this variation (which was not due to extraction or chromatography), even in carefully designed experiments.

Results

Time-course of pyrrolizidine alkaloid storage and pyrrolizidine alkaloid transformation in larvae

Larvae of *C. transiens* (last instars, *i.e.* L 7) were each fed with 1, 2 and 3 mg of the monoester 7*S*-heliotrine or one of the cyclic diester senecionine or seneciphylline, respectively. In a parallel experiment the same amounts of pyrrolizidine alkaloids were administered by injection into the haemolymph. 48 h later the larvae were collected and dissected. After alkaloid extraction the tissue distribution of pyrrolizidine alkaloids was determined by capillary GLC. As can be seen from Table I and Fig. 1 A–C (here, only 2 mg data are illustrated), all three pyrrolizidine alkaloids are predominantly stored in the larval integument, confirming earlier results with 7*S*-heliotrine [9].

Pyrrolizidine alkaloid storage was positively correlated with initial alkaloid input (Table I). If the alkaloid dose exceeded 2 mg, then part of the diester alkaloids was no longer resorbed and stored, but eliminated with the faeces (Table I B, C). The monoester 7*S*-heliotrine was usually completely resorbed (Table I A), as described in [1].

Results after pyrrolizidine alkaloid injection were similar to those after pyrrolizidine alkaloid feeding (Table I, Fig. 1), but faecal elimination was always higher than that after oral application, indicating the participation of the Malpighian tubule system in alkaloid elimination. In all these experiments only part of the pyrrolizidine alkaloids applied could be recovered (ranging from 21 to 41% after injection, and 25–56% after oral application in experiments with 3 mg pyrrolizidine alkaloid). We assume that the difference is due to degradation of 7*R*- and 7*S*-heliotrine, senecionine and seneciphylline to non-alkaloidal compounds, which could not be detected by our chromatographic methods.

In another series of experiments the time-course of pyrrolizidine alkaloid storage and pyrrolizidine alkaloid metabolism was studied employing 7*S*-heliotrine: Last instar larvae individually obtained 3 mg 7*S*-heliotrine either on a small piece (0.7 × 0.7 cm) of a *Senecio* leaf, which was completely eaten within 15 min or by injecting the same amount of 7*S*-heliotrine. Within a 48 h period animals and their faeces were collected at intervals between 2 and 12 h (Fig. 2–5). Larvae were immediately dissected into their main organs, such as guts, haemolymph, and integument. Alkaloids were extracted and analyzed by capillary GLC.

After oral application of 7*S*-heliotrine, the pyrrolizidine alkaloid content of the gut remained rather low and further decreased within the 48 h period (Fig. 2 A, B), whereas substantial amounts of pyrrolizidine alkaloids occurred in the haemolymph within the first 24 h, which decreased continuously during the following day. From the haemolymph the pyrrolizidine alkaloids were apparently transferred to the integument which, after 48 h, contained between 75% (females) and 90%

Table I. Dose-dependent distribution of the PA monoester heliotrine (A) and the diesters senecionine (B) and seneciphylline (C) in L 7 larvae of *Creatonotos transiens*. The alkaloid contents of the isolated tissues were determined by capillary GLC. Further details see Fig. 1. ○ = PA applied oraly, i = PA injected.

	PA dose [mg]	PA content/tissue [µg]													
		Faeces		Haemol.		Fat body		Sex organs		Gut		Integument		Total	
		○	i	○	i	○	i	○	i	○	i	○	i	○	i
A. Heliotrine	1	0	22	6	0	3	0	0	0	0	0	166	462	175	484
	2	0	33	16	49	9	2	0	0	4	8	583	675	612	768
	3	0	177	108	440	37	30	0	0	14	37	809	662	968	1346
B. Senecionine	1	5	115	1	4	2	6	0	0.3	1	2	276	219	285	376
	2	6	190	6	2	4	3	0	0	1	2	335	303	352	501
	3	295	349	3	12	4	11	0.1	0	3	8	297	324	602	704
C. Seneciphylline	1	8	173	7	6	1	6	0.1	4	1	11	240	356	257	556
	2	102	302	3	24	5	23	0	3	8	8	363	510	481	870
	3	189	368	14	7	31	4	0	3	8	6	629	246	871	634

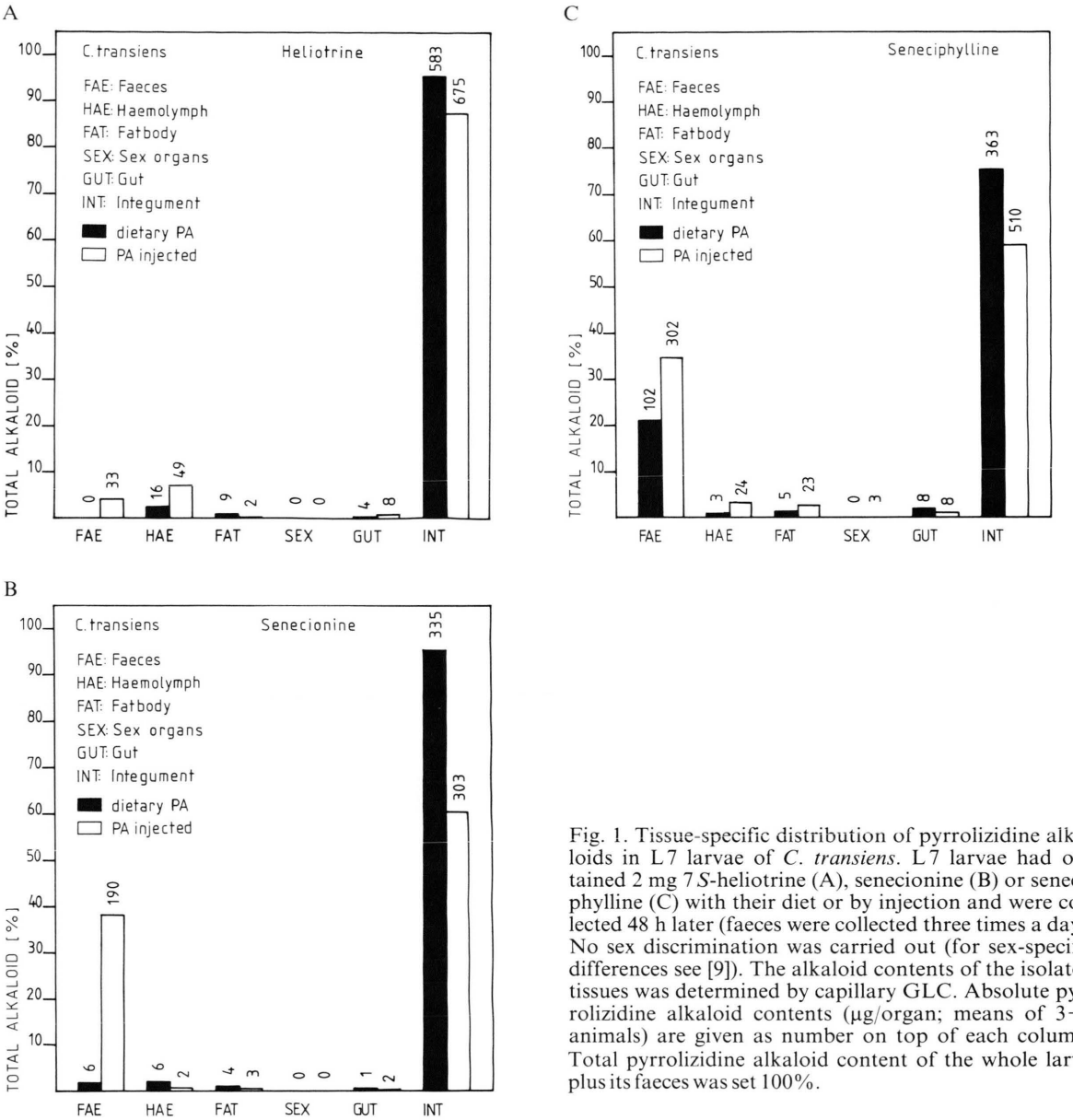

Fig. 1. Tissue-specific distribution of pyrrolizidine alkaloids in L7 larvae of *C. transiens*. L7 larvae had obtained 2 mg 7*S*-heliotrine (A), senecionine (B) or seneciphylline (C) with their diet or by injection and were collected 48 h later (faeces were collected three times a day). No sex discrimination was carried out (for sex-specific differences see [9]). The alkaloid contents of the isolated tissues was determined by capillary GLC. Absolute pyrrolizidine alkaloid contents (μg/organ; means of 3–5 animals) are given as number on top of each column. Total pyrrolizidine alkaloid content of the whole larva plus its faeces was set 100%.

(males) of the total alkaloids (Fig. 2A, B). In principle, a similar time-course of pyrrolizidine alkaloid distribution was observed when 7*S*-heliotrine was administered by injection (Fig. 3A, B). However, the relative amounts found in the faeces (especially in females) were higher, indicating that part of the pyrrolizidine alkaloids were removed from the haemolymph *via* the Malpighian system.

The stereochemical configuration of the hydroxyl group at C7 of 7*S*-heliotrine is inverted to 7*R*-heliotrine in larvae of *C. transiens*. (Both compounds can be separated by capillary GLC [10].) The stereochemical inversion of 7*S*-heliotrine to 7*R*-heliotrine reached a plateau after 2 days (Fig. 4A, B). There was a remarkable bias towards males, which processed 7*S*-heliotrine at a much higher rate (plateau between 30–50% in other experiments of which data are not shown) than females. No difference was seen between feeding and injection experiments, indicating that the gut tissue

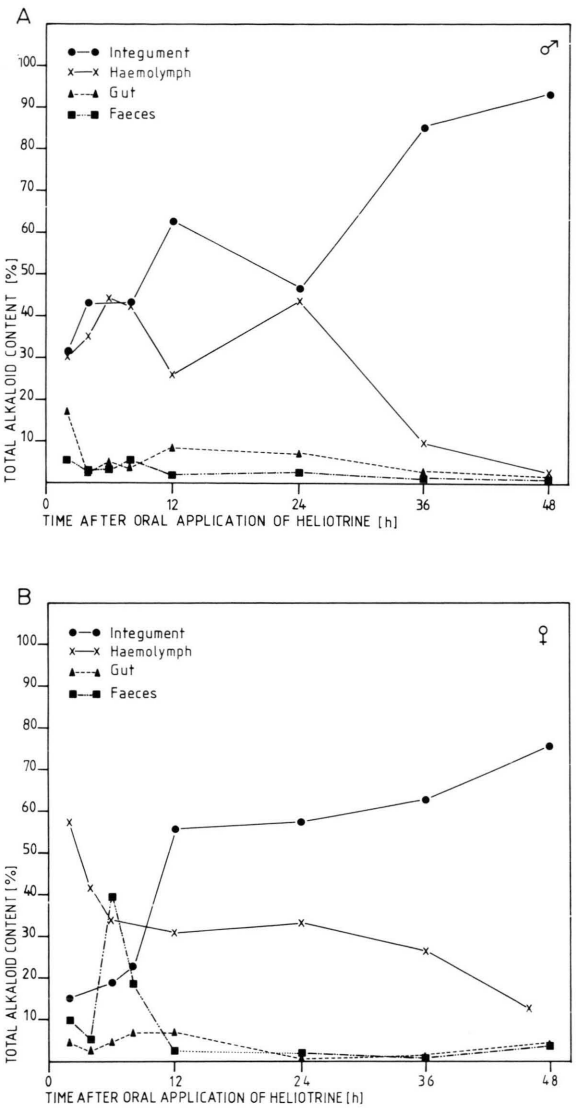

Fig. 2. Tissue-specific time-course of pyrrolizidine alkaloid (7*S*/7*R*-heliotrine) storage in *C. transiens* after oral application. A, males; B, females. L 7 larvae were reared on the semiartificial diet and were supplied with 3 mg 7*S*-heliotrine. At the time intervals shown, the faeces produced were collected and the corresponding larvae were dissected into their main organs. The sum of pyrrolizidine alkaloids of all organs plus faeces was set 100%. For sake of lucidity only the relative pyrrolizidine alkaloid contents of integument, haemolymph, gut, and faeces are shown.

was not the site of 7*S*-heliotrine inversion (Fig. 4B). In none of these two parallel experiments the amount of 7*R*-heliotrine in the faeces fraction exceeded the range of 0 to 6.1%.

Both 7*S*- and 7*R*-heliotrine were predominantly present in larvae and imagines of *C. transiens* as their N-oxides [9, 11]: The time-course of biotransformation of 7*S*-heliotrine into 7*S*- or 7*R*-heliotrine-N-oxide is illustrated in Fig. 5. Haemolymph and integument contained more than 60% of the total pyrrolizidine alkaloids that was present in a larva 2 h after feeding (comp. Fig. 2); at that time already 90 to 100% of heliotrine had

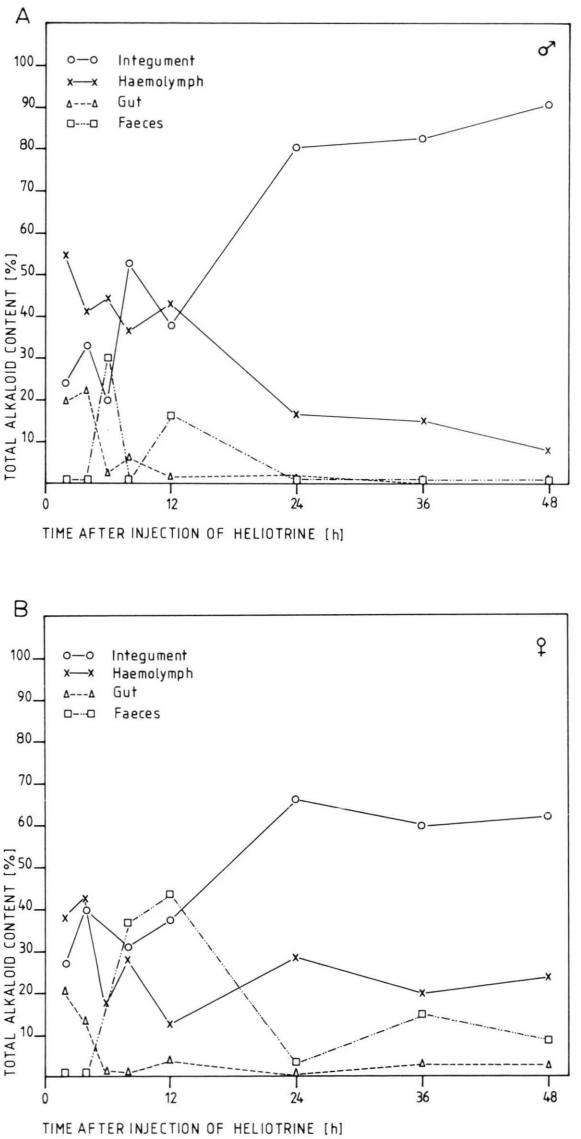

Fig. 3. Tissue-specific time-course of pyrrolizidine alkaloid storage after injection of 3 mg 7*S*-heliotrine into L 7 larvae. A, males; B, females. For details see Fig. 2.

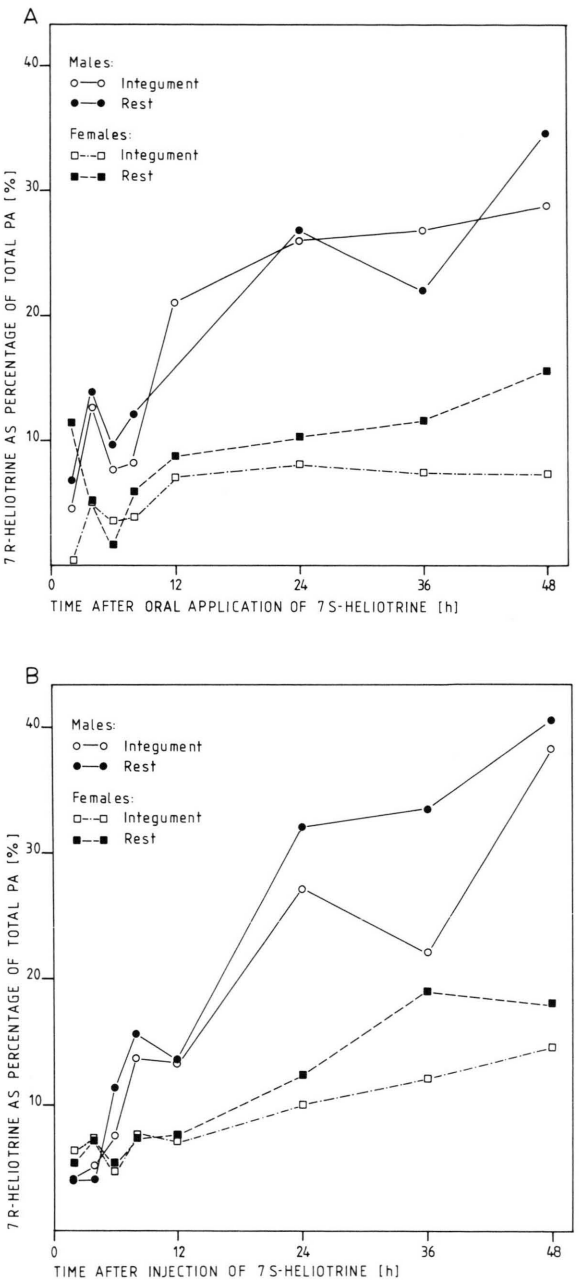

Fig. 4. Tissue-specific time-course of the biotransformation of 7 *S*-heliotrine to 7 *R*-heliotrine in L 7 larvae of *C. transiens*. A, after oral application; B, after injection of 3 mg 7 *S*-heliotrine. Feeding regimes and extraction were performed as in Fig. 2. For lucidity only the data for the integument and all the remaining tissues ("Rest") are illustrated. Faeces are excluded. 7 *S*- and 7 *R*-heliotrine can be separated and quantified by capillary GLC [10]. The amounts of both compounds in a given fraction was set 100%; the relative abundance of 7 *R*-heliotrine is illustrated in the graph (in %).

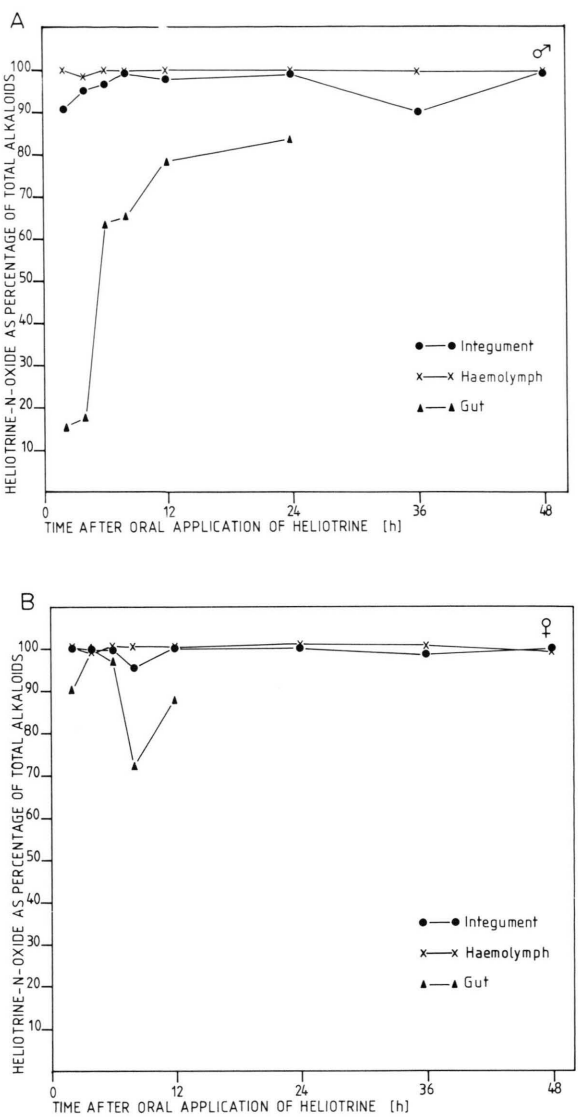

Fig. 5. Tissue-specific time-course of pyrrolizidine alkaloid-N-oxide formation in *C. transiens* after oral application of 7 *S*-heliotrine. A, males; B, females. The feeding regime and tissue preparation was carried out as in Fig. 2. Determination of pyrrolizidine alkaloid-N-oxides: Homogenates were divided in two samples of equal volume. One sample was treated with powdered zinc to reduce pyrrolizidine alkaloid-N-oxides. The other sample was directly extracted. Pyrrolizidine alkaloid contents of both samples were determined by capillary GLC, which were used to calculate the percentage of pyrrolizidine alkaloid-N-oxide in the total alkaloid content (= 100%) of a given tissue/organ.

been converted into its N-oxide (Fig. 5). Data after injection were nearly identical to those displayed in Fig. 5 and are therefore not shown. The N-oxide formation in the gut tissues was much slower (at least in males) than in the haemolymph or integument.

Fate of pyrrolizidine alkaloids during pupal development and at eclosion

Pyrrolizidine alkaloids were not lost with larval or pupal exuviae or the lightly-built cocoon (Table II). After eclosion pyrrolizidine alkaloids were redistributed in *C. transiens:* When adults were decapitated after eclosion they quantitatively ejected up to 100 µl of meconium: males (n = 10) with a mean weight of 469 mg produce 75 mg meconium, females (n = 10) with a mean weight of 569 mg 44 mg meconium, respectively. Up to 17% of total pyrrolizidine alkaloid found in the animal could be recovered from the meconium; its concentration could be as high as 2.9 mM (Table II).

Time-course of pyrrolizidine alkaloid processing after eclosion

Males

In males, up to 13% of stored heliotrine is present in the corema one day before eclosion, which is apparently then converted into the pheromone, 7 *R*-hydroxydanaidal [9]. We have now analyzed the alkaloid and pheromone contents in males, which had obtained 3 mg 7 *S*-heliotrine as last instar larvae, over a 48 h and 120 h period after eclosion (Fig. 6 A, B). Parallel to a decrease in pyrrolizidine alkaloid content of the body (mainly integument), an increase of 7 *R*-hydroxydanaidal over the first 36 h was observed (Fig. 6). Later on the pheromone level remained almost stable whereas the content of pyrrolizidine alkaloids decreased further (Fig. 6 B). The degree of decline in pyrrolizidine alkaloids and of pheromone production varied between experiments, in general 10–30% of pyrrolizidine alkaloids were converted into 7 *R*-hydroxydanaidal.

Females

The data indicate that in females, pyrrolizidine alkaloids were partly (50–80%) removed from the integument and transferred to the ovaries and subsequently to the eggs, which could contain up to 4700 µg pyrrolizidine alkaloids/g f.w. (Table III B II). This redistribution took place within the first 48 h after eclosion (Fig. 7 A, B). The absolute transfer rate between integument and ovaries differed between experiments, due to unknown reasons.

Table II. Fate of 7 *S*-heliotrine in *Creatonotos transiens*. Larvae were kept on a semiartificial diet which was supplied with a total amount of 2 or 3 mg 7 *S*-heliotrine. Meconium, exuviae, cocoons and imagines were analyzed for pyrrolizidine alkaloid content by capillary GLC. Meconium was obtained quantitatively by decapitating the animals shortly after eclosion and collected in Eppendorf vials.

Animal No.	PA fed [mg]	PA content [µg/animal]				
		Imagines	Meconium	Exuviae		Cocoon
				Larval	Pupal	
Males						
1.	2	439	92	3	1	1
2.	2	902	87	3	0	3
3.	2	552	35	0	3	0
4.	3	1305	6	0	1	0
5.	3	1098	7	0	0	0
6.	3	685	10	0	1	0
Females						
1.	2	1021	4	2	0	3
2.	2	901	31	1	1	1
3.	2	883	13	7	0	4
4.	2	970	20	2	0	0
5.	3	1539	2	0	8	0
6.	3	1001	22	2	0	1

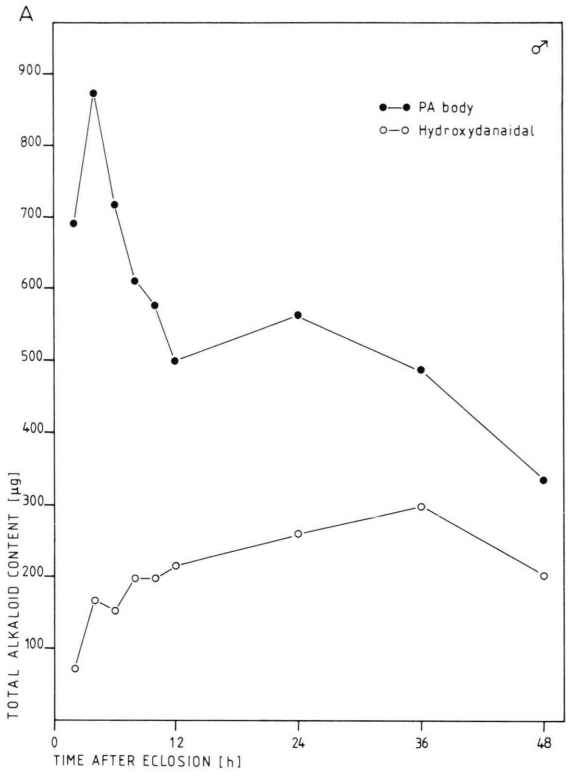

Fig. 6. Pyrrolizidine alkaloid storage and pheromone formation in males (not mated) after eclosion. A, within a 48 h period; B, within a 120 h period (two independent experiments). L7 larvae obtained 3 mg 7*S*-heliotrine with the diet. Prior to eclosion, which was monitored for each animal, pupae were sexed. At the time intervals shown, animals were killed and dissected. In males, coremata were excised and analyzed separately for their 7*R*-hydroxydanaidal content.

Table III. Parental endowment of eggs with acquired plant alkaloids. Larvae were reared on the semiartificial diet and either been fed 3 mg 7*S*-heliotrine or no pyrrolizidine alkaloids at all. After eclosion the imagines were sexed and either combined as single pairs (A) (here, only the males contained pyrrolizidine alkaloids, the females were PA-free) or as groups of 5 pairs per cage (B). In the latter experiment, 4 groups of PA-rich males were mated with PA-free females (I); in 5 groups only the females were PA-rich (II); in a third test, both partners were PA-rich (III). The number of copulations was not recorded. After 5 d the experiment was terminated. By that time the females had finished egg-laying, and only the fertile eggs were collected and weighed. PA contents of fertile eggs, males and females were determined by capillary GLC.

A. Single pairs	PA content (µg/animal or clutch)*		
	Male	Female	Eggs
1.	211	32	51
2.	212	89	14
3.	327	141	15
4.	99	253	36
5.	77	61	6
6.	141	20	15
Mean	178	99	23

B. 5 Pairs/cage	PA content (µg/g f.w.) eggs**
I. Only males with pyrrolizidine alkaloids	
1.	1624
2.	1052
3.	815
4.	1743
Mean	1309
II. Only females with pyrrolizidine alkaloids	
1.	4479
2.	4763
3.	4067
4.	3655
5.	3260
Mean	4045
III. Both females and males with pyrrolizidine alkaloids	
1.	4480
2.	6443
Mean	5462

* Pyrrolizidine alkaloid contents were determined after copulation.

** Pyrrolizidine alkaloid content/egg 0.3 (I) to 1.3 µg (III); mean weight/egg was 194 µg (n = 3).

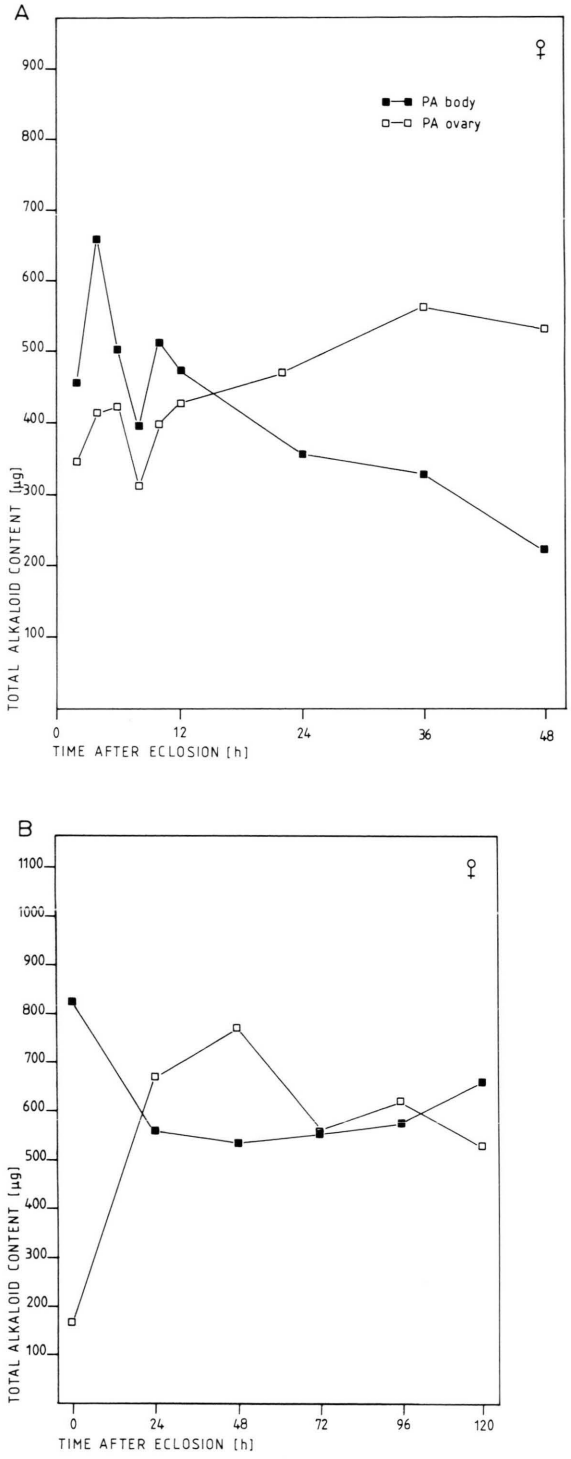

Fig. 7. Redistribution of pyrrolizidine alkaloids in virgin females after eclosion. A, within a 48 h period; B, within a 120 h period (two independent experiments). Feeding regimes and extractions were performed as in Fig. 6. At the time intervals shown, females were killed and their ovaries (with eggs) removed.

We analyzed the pyrrolizidine alkaloid content of the mating partners which had or had not consumed pyrrolizidine alkaloids in relation to the alkaloid levels of the produced eggs (Table III). Both partners, but also males and females alone, could contribute to the pyrrolizidine alkaloid content of the eggs: About 40% of the pyrrolizidine alkaloids stored by the male were found to be transferred (Table III A). The female's endowment was about 3 times higher than that of the male's, which can only transfer pyrrolizidine alkaloids to the female *via* its spermatophore during copulation.

Influence of larval alkaloid ingestion on corema size, pheromone and alkaloid content

The development of male scent organs (coremata) is known to be quantitatively dependent upon the amount of pyrrolizidine alkaloids eaten by the larvae [12, 13]. The expression of this morphogenetic effect depends on the availability of ecdysone. The sensitivity to induce the growth of the corema anlage ends in the prepupal stage [14].

In a series of four experiments we tried to find out in which larval stage the pyrrolizidine alkaloid uptake was most important as a condition for corema induction, pheromone formation and alkaloid storage. Food uptake and larval growth are tabulated in Table IV.

Table IV. Food and alkaloid uptake during larval development. Larvae were kept continuously on *Senecio jacobaea* leaves (from non-flowering plants) and could obtain food *ad libitum*.

Larval stage	Duration [d]	Food intake [mg]	PA uptake [µg]	Larval weight* [mg]
L1	4	38	2	1.6
L2	4	44	2	12
L3	4	237	10	44
L4	4	756	33	191
L5	4	1,867	79	430
L6	4	2,877	123	900
L7	6	6,714	285	1,563
Total	30	12,532	534	

* These larvae were reared on the semiartificial diet which gave similar results as the plant diet.

In a first experiment we kept larvae on the pyrrolizidine alkaloid-containing plant, *Senecio jacobaea,* from L 1 to L 7, from L 2 to L 7, from L 3 to L 7 *etc., i.e.* larvae always obtained pyrrolizidine alkaloids in their L 7 stage. As can be seen from Fig. 8, corema development, which is measured by weight and length, was equally high in all feeding regimes, even in those animals which had obtained pyrrolizidine alkaloids in L 7 only. 7 *R*-Hydroxydanaidal content was about 60 µg/male. Alkaloid storage was between 180 and 210 µg/male.

In a second experiment larvae obtained *Senecio* from L 1 to L 7, from L 1 to L 6, from L 1 to L 5 *etc.* (Fig. 9). Corema size and weight dropped substantially if larvae did not obtain pyrrolizidine alkaloids in later larval stages, especially L 5 to L 7. This effect was even more pronounced for 7 *R*-hydroxydanaidal and total pyrrolizidine alkaloid content: If L 7 alone was left out from pyrrolizidine alkaloid feeding, pheromone and pyrrolizidine alkaloid content were reduced by 80–90% as compared to animals which had *Senecio* from L 1

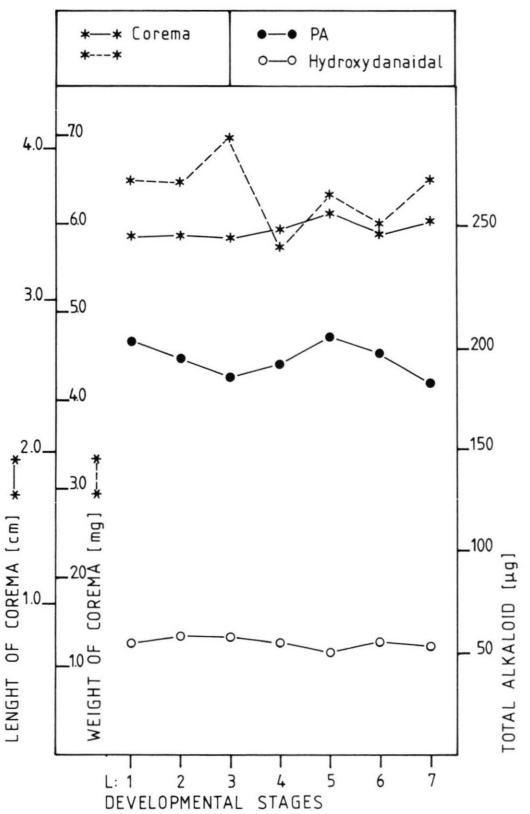

Fig. 8. Influence of larval stage on corema induction, pheromone production and alkaloid storage. Larvae were reared on the semiartificial diet or obtained pyrrolizidine alkaloid containing *Senecio jacobaea* leaves according to the feeding regime (for details see text). Data for L 7 mean: pyrrolizidine alkaloid containing food in L 7; L 6 = pyrrolizidine alkaloids in L 6 and L 7, L 5 = pyrrolizidine alkaloids in L 5–L 7, etc. About 14 animals were analyzed for every single feeding experiment. Corematal size (the longer branch was measured) and weight (complete corema), pyrrolizidine alkaloids and 7 *R*-hydroxydanaidal contents of imagines were determined 6 h after eclosion.

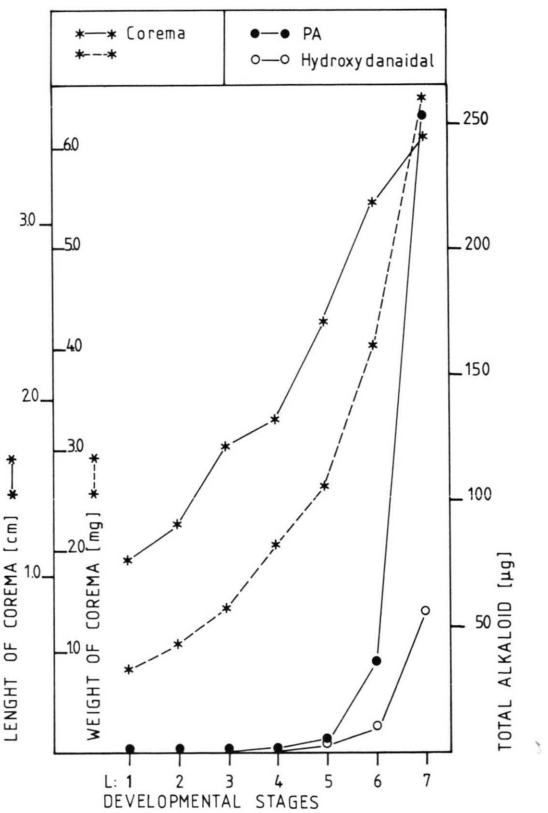

Fig. 9. Influence of larval stage on corema induction, pheromone production and alkaloid storage. Experimental design similar to that in Fig. 8, however: L 7 = pyrrolizidine alkaloids from L 1 to L 7, L 6 = L 1–L 6, L 5 = L 1–L 5, etc.

to L 7 (Fig. 9). Intake of food and pyrrolizidine alkaloids, however, only amounted to 50% in the L 7 stage (Table IV).

The importance of the L 7 stage for pyrrolizidine alkaloid accumulation and pheromone production [13] was even more evident in a third experiment, where larvae had access to *Senecio* in a single life stage only, *i.e.* either in L 1, or L 2 or L 3 *etc.* (Fig. 10). Full-sized coremata were only obtained if larvae fed on *Senecio* in L 6 or L 7, although some induction was seen in larvae which had pyrrolizidine alkaloids in their L 4 or L 5 stages (Fig. 10). Only in L 6 and L 7 alkaloid and pheromone content were increased substantially (Fig. 10).

The importance of the L 7 stage for corema induction, pyrrolizidine alkaloid and 7 *R*-hydroxydanaidal accumulation was confirmed in a fourth set of experiments in which L 7 larvae, which had been reared pyrrolizidine alkaloid-free, were allowed to feed on *Senecio* for a limited time only, ranging from 2 h to 12 h. As can be seen from Fig. 11, even short periods of pyrrolizidine alkaloid ingestion had a marked effect and resulted in large coremata (full-size after 6 h feeding). In contrast to Fig. 8–10 the levels of 7 *R*-hydroxydanaidal were higher than those of pyrrolizidine alkaloids, but were altogether much lower than in the above experiments, since the overall amounts of pyrrolizi-

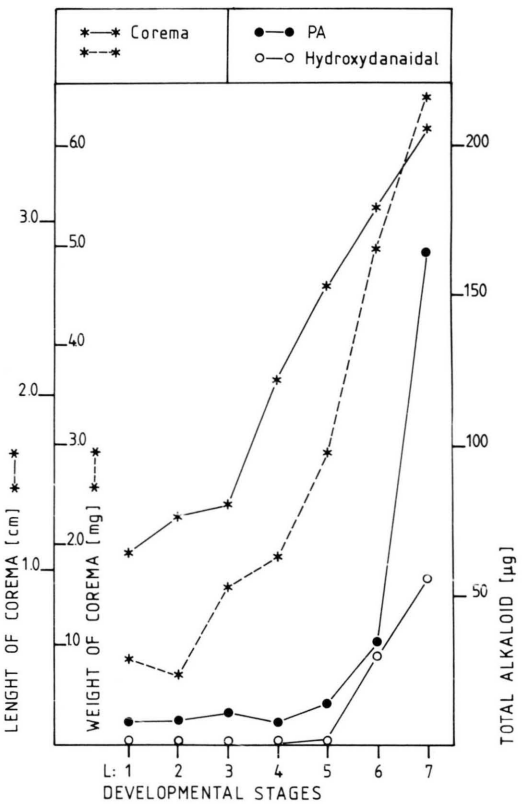

Fig. 10. Influence of larval stage on corema induction, pheromone production and alkaloid storage. Feeding regime and experimental design see Fig. 8 and text. L 7 = pyrrolizidine alkaloids in L 7 only, L 6 = pyrrolizidine alkaloids in L 6, etc.

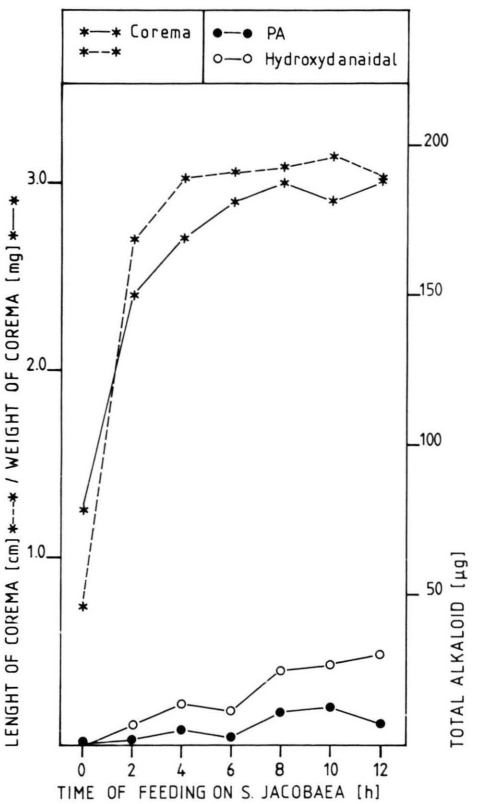

Fig. 11. Influence of short-term feeding of the pyrrolizidine alkaloid plant *Senecio jacobaea* on corema induction, pheromone production and alkaloid storage. L 7 larvae, which had been reared pyrrolizidine alkaloid-free on the semiartificial diet were allowed to feed on *S. jacobaea* leaves for the time intervals shown. Afterwards they were put back to pyrrolizidine alkaloid-free diet. Corematal weight and length, pyrrolizidine alkaloid and 7 *R*-hydroxydanaidal contents of males were determined 6 h after eclosion.

dine alkaloids ingested were small. For maximal pyrrolizidine alkaloid storage and pheromone production a longer feeding time was necessary (Fig. 8–10).

In the feeding regimes of Fig. 8–10, we also analyzed the alkaloid contents of females and found that they accumulated about 2 to 3 times as many pyrrolizidine alkaloids than males (data not shown).

Discussion

Pyrrolizidine alkaloid storage in the integument

All three pyrrolizidine alkaloids (a monoester as well as two diester alkaloids), which were applied either by diet or by injection, predominantly accumulate in the larval integument within days. The storage of plant-derived allelochemicals and of chemical defence compounds, synthesized by insects themselves, in the integument has also been reported in other cases and might be a general phenomenon. Examples are cardenolides in danaine butterflies [20], in a ctenuchid moth *(Syntomeida epilais)* [1, 21], in the milkweed bug *Oncopeltus* [22] and a chrysomelid beetle [23]; cyanogenic glycosides in zygaenid moths [24]; salicyl aldehydes in chrysomelid beetles [25]; flavonoids in a butterfly [26]; pyrrolizidine alkaloids in a chrysomelid beetle [27], quinolizidine alkaloids in a pyralid moth *(Uresiphita reversalis)* [28, 29] and gallic acid in a locust *(Anacridium melanorhodon)* [39]. Where and how pyrrolizidine alkaloids are stored in the integument of *Creatonotos* is an unsolved problem as yet. The storage of acquired or endogenous defence compounds in the integument may be ecologically important (it is the first tissue which a predator encounters) or physiologically advantageous (because the allelochemicals are thus far away from internal organs and do not intoxicate the animals themselves).

N-Oxide formation

Free pyrrolizidine alkaloid bases were converted within hours to their N-oxides, which constituted more than 90% of the pyrrolizidine alkaloid stored in *Creatonotos* (Fig. 5, [9]). The conversion took place probably in all tissues. The underlying enzymatic mechanism and localization have not been studied so far. The storage of pyrrolizidine alkaloids as their N-oxides is a widespread phenomenon in plants [15] but has been also recorded from other pyrrolizidine alkaloid sequestering arctiids [16, 17]. Pyrrolizidine alkaloid-N-oxides are polar molecules which cannot pass biomembranes by simple diffusion, whereas the free bases are more lipophilic and can diffuse at alkaline pH to some degree. It can be speculated that the conversion of pyrrolizidine alkaloids to their N-oxides support the cell in restricting these molecules to special storage compartments.

7 S-Heliotrine to 7 R-heliotrine

7 *S*-Heliotrine was substantially converted to 7 *R*-heliotrine within 48 h (Fig. 4, [10]). This effect was more pronounced in males than in females, which seems plausible since the males can directly exploit only the 7 *R*-heliotrine for the biosynthesis of 7 *R*-hydroxydanaidal [10, 18, 19]. The ability for this biotransformation is not universally present in moth larvae, but seems to be restricted to pyrrolizidine alkaloid exploiting species, such as *Creatonotos* and a few other Arctiidae (M. Wink, E. v. Nickisch-Rosenegk, and D. Schneider, unpublished). This can be interpreted as taking even advantage of the few naturally occurring pyrrolizidine alkaloids which are 7 *S*-configurated while the majority shows 7 *R*-configuration anyway.

Meconium

Lepidoptera release some of their meconium shortly after eclosion, especially when disturbed. The main components seem to be ommochromes, which have been considered as waste products [30]. In *Creatonotos* the meconium can additionally contain reasonable amounts of pyrrolizidine alkaloids (Table II), which may function there as a defence secretion. But the ommochromes could also play a role as deterrents. Lepidoptera are very soft and vulnerable shortly after eclosion; a chemical defence secretion which may be sprayed onto a predator would be a very useful acquisition.

Pyrrolizidine alkaloid content of eggs and pyrrolizidine alkaloid transfer from males to females

Within 2 d after eclosion females transferred 50–80% of the pyrrolizidine alkaloids which were so far stored in the integument to the ovaries and subsequently to the eggs (Fig. 7, [11]). The pyrroli-

zidine alkaloid content of eggs reached on average 4 mg/g f.w. (equivalent to 16 mM), a concentration which is likely to be deterrent for predators. The allocation of alkaloids to the eggs has been described from other species, such as *Utetheisa ornatrix* [31]. Additionally for this species it has been shown that males can donate dietary allelochemicals to their mates by way of the spermatophore. Thus the males' pyrrolizidine alkaloids would eventually find their way into the eggs. We have now also observed this phenomenon in *C. transiens* (Table III). A biparental defensive endowment of eggs with acquired alkaloids might therefore be a more widespread feature [31, 37, 38]. Since most *Creatonotos* moths are short-lived they no longer need defensive pyrrolizidine alkaloids for themselves after copulation and egg-laying. An endowment to the eggs would increase their reproductive success and would thus be ecologically advantageous [31].

Pyrrolizidine alkaloid conversion to pheromone

7 *R*-Hydroxydanaidal which has been considered as a male pheromone in many arctiid and danaine butterflies [32–36], was synthesized within 48 h after eclosion of *C. transiens* (Fig. 6). It seems plausible to assume that 7 *R*-hydroxydanaidal derives from dietary pyrrolizidine alkaloids, since 7 *R*-hydroxydanaidal content and dietary pyrrolizidine alkaloids were generally well correlated [5, 12, 13, 33]. Pyrrolizidine alkaloid feeding in the last larval stage, *i.e.* L 7 is apparently most important for the pheromone production (Fig. 8–11): larvae take up about 50% of pyrrolizidine alkaloids (complete development = 100%) alone during their last larval stage (Table IV). Usually pyrrolizidine alkaloid storage was much higher than respective pheromone contents (Fig. 8–10). We cannot explain why pheromone production was enhanced over pyrrolizidine alkaloid storage during short-term feeding in the L 7 stage (Fig. 11).

Pyrrolizidine alkaloids as a morphogen for corema development

Male danaine butterflies and many arctiid moths have abdominal androconial scent organs which are employed to dissipate the male pheromones [12, 40]. (Both males and females of *C. transiens* were shown to possess sensor cells for these pheromones on their antennae [7].) *Creatonotos* is remarkable in that the size of its corema depends on dietary pyrrolizidine alkaloids: without pyrrolizidine alkaloids only small corema with few hairs were formed [5–7, 12, 13]. Pyrrolizidine alkaloids thus act as a morphogen (with a dependence on ecdysone [14]) for the development of the corema anlage. The inducing activity ended in the prepupal stage [14]. Our feeding regimes clearly showed that an acquisition of pyrrolizidine alkaloids in the L 6 or even better in the L 7 stage was most critical for the full development of the corema (Fig. 8–11). Even feeding on a pyrrolizidine alkaloid plant for a few hours during L 7 could be sufficient for nearly full-sized scent organs (Fig. 11), whereas pyrrolizidine alkaloid ingestion in earlier stages never led to full-sized coremata. For maximal pyrrolizidine alkaloid storage in the integument, longer feeding times (days not hours) are necessary in L 7.

Open questions

Male moths of *C. transiens* which were caught in the field had large coremata and high 7 *R*-hydroxydanaidal contents, but were nearly or totally devoid of pyrrolizidine alkaloids [11]. This result is surprising in view of our laboratory experiments where large coremata and high 7 *R*-hydroxydanaidal contents were always accompanied by high pyrrolizidine alkaloid contents [9] (Fig. 8–10). How to explain the discrepancy? Our knowledge on the natural food plants of *Creatonotos* is scanty but larvae were observed to feed on *alkaloid-free* plants, such as rice, other Gramineae or cabbage [6]. This could be the reason for the lack of pyrrolizidine alkaloid storage in the field-caught moths which we had analyzed (females, too, did not contain pyrrolizidine alkaloids). But how to explain the high pheromone levels and large coremata of the males? Are we still overlooking one (or the) critical factor (with or without pyrrolizidine alkaloids) in the chain of processes of the corema induction and pheromone production? Alternatively, we know from Fig. 11 that even short-term feeding on a pyrrolizidine alkaloid plant during L 7 may result in large coremata and reasonable pheromone levels but low pyrrolizidine alkaloid contents. Some arctiid larvae (*Creatonotos,* too?) are known to wander around in their habitat and feed on different plants [41]. Since they must have

receptors for pyrrolizidine alkaloids [2] one could argue that such mobile larvae search for pyrrolizidine alkaloid plants. But since they feed for a short time only the amount then acquired just suffices to induce corema formation and pheromone production, but is not enough for the storage of pyrrolizidine alkaloids. It is obvious that thorough field studies on the diet and feeding habits of this species are necessary to clarify the biochemical and morphogenetic discrepancies encountered. Equally important but not discussed in this paper would be field studies aimed to elucidate the behavioural puzzle of the male-male-female assemblies (so-called leks [6, 7, 33]) which have not yet been seen outdoors but only in captivity.

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