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A Pheromone Precursor and Its Uptake in Male *Danaus* Butterflies

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Summary. 1. Male *Danaus chrysippus* butterflies the larvae of which have been raised indoors on their natural foodplants (*Asclepias*) lack the pyrrolizidinone pheromone (Fig. 1, I) which is known from the hairpencils of field-caught butterflies.

2. Male *D. chrysippus* have been observed actively approaching withered parts of a *Heliotropium* plant in Kenya. These observations could be repeated in the laboratory. On the plants, the males suck extensively.

3. A pyrrolizidine alkaloid (Fig. 1, II) has been isolated and characterized as lycopsamine from this *Heliotropium* species.

4. Significant and up to "normal" amounts of I are found in the hairpencils after the indoors-raised males were allowed to: a) suck on withered and remoistened *Heliotropium*, or b) feed on a methanol extract of *Heliotropium*, or c) feed on a solution of alkaloid (II) isolated from *Heliotropium* (see Table 1, Fig. 2). It therefore appears that substance II is a dietary precursor of I.

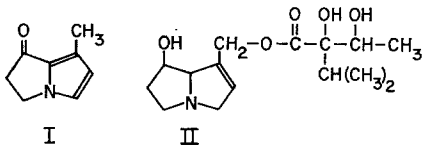
5. Electroantennogram recordings revealed the presence of antennal olfactory receptors for both substances I and II, as well as for the odor of the withered and remoistened *Heliotropium* (Fig. 3).

6. Experiments in which radiolabelled compounds were administered to *D. gilippus berenice* males also suggest that the pyrrolizidine pheromone (I) is biosynthesized from an exogenous alkaloid precursor.

Introduction

The existence of a male sexual pheromone (arrestant scent, aphrodisiac) has been established in the Florida Queen butterfly, *Danaus gilippus berenice* Cr. by Pliske and Eisner (1969). This compound, a volatile pyrrolizidinone (Fig. 1, I) (Meinwald *et al.*, 1969) is found in abdominal brushes ("hairpencils") which are exposed during a phase of courtship behavior (see Brower *et al.*, 1965); it is perceived by antennal olfactory receptors (Schneider and Seibt, 1969; Myers and Brower, 1969). Behavioral studies indicate that the mating success of pyrrolizidinone-deficient male Queens is low compared to that of normal males (Pliske and Eisner, 1969).

The occurrence of this pyrrolizidinone, or of closely related heterocyclic compounds, in a number of Danaidae of all three subfamilies (Danainae, Lycoreinae, Euploeinae) suggests that these substances play a similar role in most of these species (for ref. see Meinwald *et al.*, 1974; Edgar *et al.*, 1971, 1973; Schneider, 1974/75).



I II

Fig. 1. *I* Major component of *Danaus chrysippus* hairpencil secretion: 2,3-dihydro-7-methyl-1H-pyrrolizine-1-one; *II* Pyrrolizidine alkaloid (lycopsamine) found in *Heliotropium steudneri* Vatke (Boraginaceae)

The observation that the hairpencils of *D. gilippus berenice* Cr. and *D. chrysippus* L. males the larvae of which have been reared on the natural foodplants in the laboratory or greenhouse do not contain substance I (Meinwald *et al.*, 1971, 1974) drew the attention to earlier anecdotal observations of male danaid butterflies assembling and feeding on certain other plants (for ref. see Edgar *et al.*, 1971). These plant species belong to the families Boraginaceae, Leguminosae and Compositae which are known to contain pyrrolizidine alkaloids (see Bull *et al.*, 1968). It was therefore suggested that it is from these plants that the male butterflies may obtain precursors for the biosynthesis of substance I (Meinwald *et al.*, 1969; Meinwald and Meinwald, 1966; Edgar *et al.*, 1971).

The first confirmation of this hypothesis was brought forward by Edgar *et al.* (1973); three laboratory raised male *D. chrysippus petilea*, given access (in addition to sugar water) to the moistened boraginaceous plant *Heliotropium amplexicaule* Vahl over a one week period, later contained substance I in their hairpencils, while three identically raised males which were fed only with sugar water, were found to be deficient in I.

Edgar and Culvenor (1974) later found that the hairpencils of two danaid species from Queensland (N.E.-Australia) did in fact contain a pyrrolizidine alkaloid, which these authors presume to have been taken up from boraginaceous plants by the butterflies.

We here report our findings concerning the phenomena of pheromone deficiency and precursor composition, detection, and uptake in the African Monarch or Tiger butterfly, *Danaus chrysippus* f. *dorippus* L. A brief, preliminary account of these observations has recently been given in a wider context by Schneider (1974/75). The field observation which led to this study was the assembly of males of this species on withered parts of a *Heliotropium* in East Africa. This paper also incorporates a brief account of pilot experiments on the biosynthesis of I in *D. gilippus berenice* Cr.

Material

Danaus chrysippus f. *dorippus* L. was observed in the field in Kenya and brought to Seewiesen alive for further studies of the behavior, the electrical responses of the pheromone receptors, and the preparation of hairpencil extracts for chemical analysis. We also reared butterflies from eggs on the foodplant *Asclepias curassavica* L. (Asclepiadaceae) in the greenhouse (see Seibt *et al.*, 1972).

Withered and fresh plants of the species *Heliotropium steudneri* Vatke (Boraginaceae) were collected in the East African Rift Valley near Nairobi. This plant material was used in behavioral and electrophysiological experiments, and for chemical analysis.

Methods

A. Behavior

Field observations of behavior relevant to the pheromone precursor problem were made on *D. chrysippus* in Kenya (see above). These observations were complemented with field-caught and greenhouse-reared butterflies in Seewiesen. The butterflies were observed, photographed and filmed when approaching and assembling on the *Heliotropium* under different conditions. In control experiments, dry and fresh European plants which presumably also contain potential precursor alkaloids were presented together with other dry plants which have not been reported to contain such substances.

B. Laboratory Experiments

Males of greenhouse-reared *D. chrysippus* were subjected to the following feeding program:

1. Sugar water only (control group);
2. Sugar water plus water suspension of pulverized *Heliotropium*;
3. Sugar water plus methanol extract of pulverized *Heliotropium*;
4. Sugar water plus isolated pyrrolizidine alkaloid from *Heliotropium*;
5. Sugar water and free access to slightly moistened *Heliotropium* plant material.

After feeding times of between one and eighteen days, hairpencils of the animals were manually exposed and amputated. Most of the hairpencils were first used for an electro-antennogram (EAG) test to check their olfactory effectiveness (see Schneider and Seibt, 1969). After EAG treatment, the hairpencils were sealed in ampules containing CS_2 as solvent. In a number of cases, only one hairpencil of a pair was tested with the EAG and then sealed as above while the other was sealed without testing.

C. Chemistry

TLC Analysis of Hairpencil Extracts. Each sample of carbon disulfide in which hairpencils were preserved was diluted to about 200 µl, and a portion of this solution was spotted onto a thin layer chromatographic plate (Brinkmann Sil G-25 UV 254, 5 × 20 cm, 0.25 mm thickness). The plate was then spotted with three known quantities of synthetic pyrrolizidinone and developed with 4% methanol in methylene chloride. After drying under a stream of nitrogen, the plate was sprayed with a 2,4-dinitrophenylhydrazine (DNP) solution (prepared by dissolving 1.50 g of DNP in 7.5 ml of concentrated H_2SO_4 and adding this to a solution of 10 ml of water in 35 ml of 95% aqueous ethanol). When visualized in this manner, the pheromone appears as a purple spot, the color of which intensifies during the next three hours. The amount of pyrrolizidinone in the pair of hairpencils was estimated by comparing the intensity of the spot from the hairpencils with those produced by the known quantities spotted on the same plate; this method appears accurate within about ± 25%.

GC Analysis of Hairpencil Extracts. D. chrysippus hairpencils were obtained preserved in 0–40 µl of CS_2. Those samples containing less than 5 µl of solvent were diluted to 10–20 µl, and 5 µl to 15 µl portions of each sample were injected onto the GC (Varian Model 2100 chromatograph, equipped with a 5 ft × 2 mm I.D. glass column packed with 6% OV-1 on 60/80 Gas Chrom Q, and utilizing a flame ionization detector). The position of the pyrrolizidinone (I) peak in the chromatogram was ascertained by determination of the retention time of an authentic sample of I (ca. 2 min at 135° C), and was confirmed by co-injection experiments, as well as by coupled GC-MS analysis (Finnigan 9500/3300 Gas Chromatograph-Mass Spectrometer) of the hairpencil extract. Peak areas were measured from GC traces, and areas obtained from standard samples of I were used for calibration. The amount of I per insect was determined by estimating the proportion of total extractable material which was injected to obtain a chromatogram. (This estimate is the most important single source of error, and may range from −50% to +100%).

Extraction of Plant Material and Alkaloid Characterization (see Bull *et al.*, 1968). Dried leaves of *Heliotropium* (5 g) were extracted with methanol for 8 hr. The extract was evaporated to give 1.1 g of residue. This was extracted with 0.5 N H_2SO_4 and filtered (volume = 26 ml).

Half of the solution was brought to pH 9.4 with ammonium and extracted four times with chloroform. The extract was dried and evaporated to give 6 mg of a yellow gum. The other half of the 0.5 N H_2SO_4 solution was made up to 2 N with H_2SO_4 and stirred for 3 hr with excess zinc dust. After filtration, the reaction mixture was brought to pH 9.4 with ammonium hydroxide and extracted four times with chloroform. The extracts were dried and evaporated to give 37 mg of yellow gum. The two portions of yellow gum were demonstrated to be identical by paper chromatography and TLC.

Thin layer chromatographic analysis was carried out on silica gel G, developed with $CHCl_3$/MeOH/NH_4OH (85:15:1) and visualized with iodine, revealing one spot, r_f 0.62 (monocrotaline r_f 0.90). Paper chromatography (Whatman # 1, n-BuOH/HOAc/H_2O, 80:3:17, ascending flow, visualized with iodine) also revealed one spot r_f 0.36 (monocrotaline r_f 0.29, heliotridine r_f 0.15). The yield of alkaloid amounted to 0.24% as free base and 1.3% as N-oxide.

In its mass spectrum, the alkaloid showed significant peaks at *m/e* (rel. intensity) 299 (3), 139 (30), 138 (100), 137 (12), 95 (16), 94 (46), 93 (79), and 80 (17); a metastable peak just below *m/e* 64 corresponds to fragmentation of the molecular ion (299) to the base peak (138). Its 60 MHz nmr spectrum includes three doublets at 0.90, 0.95, and 1.25 δ (3 H each), and broad singlets at 4.83 δ (2H) and 5.91 δ (1H). Comparison of these data with those given by Bull *et al.* (1968) leads to structure II (Fig. 1). Of the eight possible diastomers accomodated by structure II, five are known to occur naturally. The precise stereochemistry of II as found in our extract, is revealed by further analysis, including hydrolysis to give a basic component which differs from heliotridine in its GC behavior and an acid component of m.p. 118–122° and $[\alpha]_D^{20} = -4.9°$ in 2N NaOH [(−) viridifloric acid] (Crowley and Culvenor, 1959), and determination of its rotation $[\alpha]_D^{20} = +2.7°$ in ethanol, to be that of the known alkaloid, lycopsamine.

Results

A. Field Behavior

In the dry season (February 14, 1973) we observed many *Danaus chrysippus* f. *dorippus* males on *Heliotropium* plants. Locality: open thornbush savannah in the East African Rift Valley south of Nairobi (see methods). Time: 9.30–11.00 a.m.; temperature 25–30° C; sunny, moderate west wind. The woody plants on which the male butterflies assembled were locally abundant and had a maximum height of 30 cm. Some plants were green with tiny yellow flowers, some dry with seeds. The butterflies approached the plants in an upwind searching flight and settled on certain dry parts, mostly near seeds, but also on dry leaves and stems. Never did we observe an approach to or sucking on green parts of the plants. On the withered plant parts they extended their proboscis, searched intensively until finding "a good place" from which they apparently sucked for several minutes. At some particulary attractive plant parts, several butterflies were found sucking in close proximity to one another. When disturbed, they flew up, and returned—again with upwind orientation—either to the same or to other plant parts, which had attracted the butterflies before. All our observations indicate that it is the odor of certain parts of the plants which is the attractive factor, but it cannot be excluded that the animals tend to approach a place where others have already settled. Optical memorisation of plant parts learned to be the preferred place must be excluded as an overall orientational means, because individual males moved over distances of up to 100 m, settling and sucking here and there. This behavior was observed along a dirt road stretch of nearly 1 km and later again over a stretch of 300 m. In both places, out of a minimum of 100 butterflies, we observed not a single female.

In order to test an earlier observation of Edgar *et al.* (1973), we killed some male *D. chrysippus,* permanently exposed their hairpencils, and put this supposed bait on the *Heliotropium* plant. Never did we observe (contrary to Edgar *et al.*, 1973) a *D. chrysippus* male paying any attention to its dead conspecific with expanded hairpencils. In some cases, the males sucked on the plant parts only centimeters away from the hairpencils of the freshly dead butterfly.

B. Greenhouse Behavior

Dry *Heliotropium* plant material was exposed in the greenhouse in Seewiesen to both sexes of freely flying wild caught and reared *D. chrysippus.* Provided there is sunshine and the temperature is above 25° C, the animals are active. They fly, court, and search for food; males (rarely females) assemble on dry *Heliotropium.* Dry *Heliotropium* (in a room with 50–60% relative humidity) has rather low attractiveness, compared to moistened plant material. When a withered plant is rubbed between the fingers, the butterflies readily suck from the skin. Fresh green *Heliotropium* was not attractive to the butterflies. The time spent with uninterrupted sucking on the plant varied from a few minutes up to twenty (sometimes even more) minutes.

In still air, the butterflies need to come very close before they settle. As in the field, they probe over the plant material with their proboscis but suck only at certain places. Leaves and upper plant parts—including seeds—are preferred, but we saw some probing and sucking from cracks of roots.

Fresh and dry European Boraginaceae (*Borago officinalis* L., *Symphytum officinale* L., *Myosotis* spec.) and Compositae (*Senecio* spec.) which are reported to contain pyrrolizidine alkaloids had no attractiveness when exposed together with *Heliotropium.* Sometimes, males walked probing on this material, but never relaxed and sucked. Control plants of other families were inspected by the males in a similar way when exposed in the neighbourhood of *Heliotropium,* however, the butterflies never sucked on them as they did on *Heliotropium.* It should be emphasized, however, that these plants were not "naturally" withered, but air dried in the laboratory.

C. Electrophysiology and Chemistry

The results of all our experiments performed in 1973/74 unequivocally show that the hairpencils of male butterflies which had fed on withered and remoistened *Heliotropium* plants, methanol *Heliotropium* extract, and isolated *Heliotropium* alkaloid itself, contained amounts of substance I significantly above control. Quantification of our results, nevertheless, meets with considerable difficulties. On the one hand, the amount of precursor ingested by the butterflies is virtually unknown, and on the other hand, the EAG method in this case is suited only for a gross quantification of the pheromone contents of the hairpencils. The reason for these difficulties is given below:

1. The EAG is a slow overall response of many odor receptors on the antenna, irrespective of their specificity to substance I or to other odorants. Since the hairpencils are known to contain variable amounts of several volatile compounds, some of which can be smelled by the human nose, hairpencils are a complex odor source (Schneider and Seibt, 1969).

Table 1. Results of feeding males of *Danaus chrysippus* with *Heliotropium* plant material and different extracts of it. Individual data are combined. Amounts of pyrrolizidinone determined by TLC analysis; detection limit ca. 0.02 μg

	No. of males	No. of hair-pencils	Food	EAG (range) (mV)	EAG (average) (mV)	Amount of subst. I (μg) per insect		
1	16	32	5% sugar solution	0.35–1.1	0.63	0.1–0.2	caged animals; fed with diet for 18–22 days	
2	6	12	5% sugar sol. + water susp. of *Heliotropium*	0.55–1.1	0.87	0.2–0.4		
3	6	12	5% sugar sol. + methanol extr. of *Heliotropium*	0.7–2.2	1.38	40.0		
4 a	4	4	withered and remoistened *Heliotropium*	2.0–2.25	2.25	12.0	animals free in the greenhouse; fed with diet for	8 days
4 b		4		[a]	[a]	16.2		
5 a	8	8		1.05–1.75	1.43	15.1		1 day
5 b		8		[a]	[a]	40.0		

[a] Sample used only for chemical analysis; no EAG measured.

2. The absolute amplitude of the EAG depends on the responsiveness of the individual antenna and the electrode position. We performed many test series with a number of female antennae. Only EAGs recorded from one antenna are directly comparable.

3. For the EAG test, the hairpencils were put into glass cartridges through which a puff of air was blown onto the antenna. In this situation, variations of the degree of expansion of the individual hairpencils—and accompanying variations in the exposure of the odor source which was to be tested—influence the EAG.

Finally, we found that the hairpencils lose some of the pyrrolizidinone in the EAG treatment (cf. Table 1, 4a vs. 4b, and 5a vs. 5b).

The results presented in the following paragraphs were selected as examples where some quantification is possible. While all other series of experiments clearly showed the same trend and overall effect, a direct comparison of the different series is not possible for the reasons given above.

Table 1 and Fig. 2 summarize the results of some of the feeding experiments. Of a total of 102 butterflies tested in several series, only 47 are presented. Table 1 gives representative information on test males fed with sugar water and with *Heliotropium* plant material or extracts of it. Data from individual males are combined.

The results show that access to a methanol extract of *Heliotropium* and to the dry plant material (Table 1, lines 3–5) brings the olfactory effectiveness and the pyrrolizidinone content of the hairpencils to levels comparable to those observed

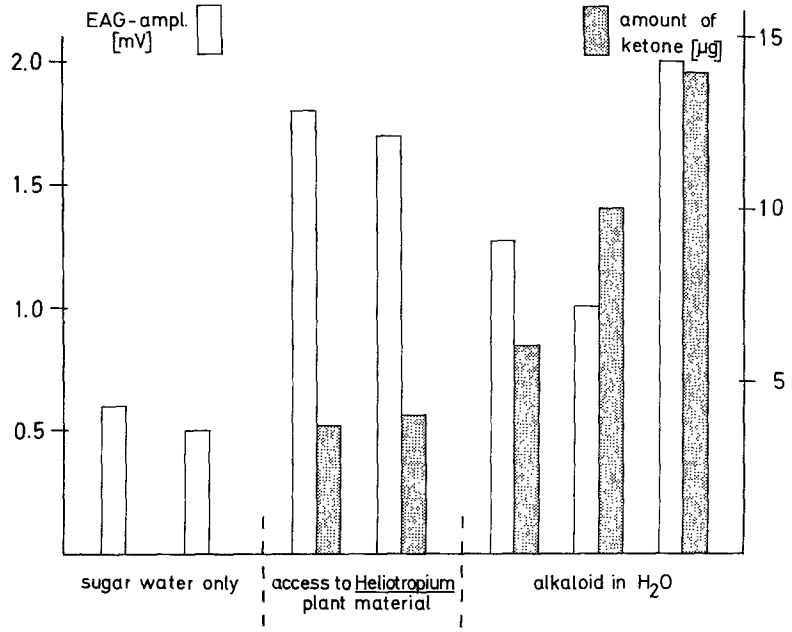


Fig. 2. EAG data and amounts of substance I from hairpencils of seven individual *Danaus chrysippus* males. Animals have been fed with different diets. Amounts of pyrrolizidinone determined by GC analysis. For details see text

n hairpencils of animals caught in the field. A comparison of line 4 with line 5 shows that after one day's access to *Heliotropium*, the hairpencils contain amounts of substance I approaching those of wild butterflies (maximally 82 µg, see Meinwald *et al.*, 1971). Here the absolute EAG amplitude increment does not compare too well to the amount of I.

The water suspension of *Heliotropium* (Table 1, line 2) slightly increases the EAG and chemical values over control. This increment, however, is not really significant.

Since the precursor contents of the different food solutions are not known these data do not allow a judgment of the effectiveness of the respective extractions.

Fig. 2 shows EAG data and amounts of substance I from hairpencils of seven individual *D. chrysippus* males. Animals fed with sugar solution alone had no detectable amounts of I (detection limit ca. 0.02 µg) and the EAG amplitude was in the range of a control air stimulus (0.5 mV).

Amounts of up to 14 µg of pyrrolizidinone were detected (gas chromatography) in hairpencils of individual butterflies fed with a solution of substance II in water. It is possible that these quantities of I, which are somewhat low compared to those found in wild males, may reflect losses suffered during EAG testing, which was conducted prior GC analyses (see Discussion). In any case, these hairpencils, as well as those of animals which had access to *Heliotropium* plant material, elicited large EAG amplitudes.

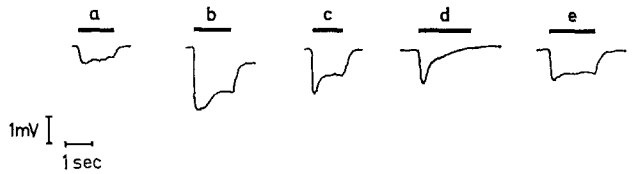


Fig. 3a—e. EAG recordings from a male *Danaus chrysippus* antenna, stimulated with (a) air alone (control), (b) 10 μg of I, (c) 10 μg of II, (d) air puff over dry *Heliotropium*, (e) air puff over moistened *Heliotropium*. Black bars mark the stimulus length. Female antennae respond similarly

Fig. 3 gives examples of an EAG recorded from an isolated antenna of a male *D. chrysippus*. It can be seen that there are receptors for the odor of *Heliotropium* plant material. Moistened *Heliotropium* (e) is a better odor source than dry *Heliotropium* (d). The pure alkaloid (II) (c) also elicits chemoreceptor responses. For comparison, the amplitudes elicited by air alone (control) (a) and substance I (b) are shown. Whether I, II and the plant exite the same receptors can only be decided by single cell recordings, since an EAG test gives only an overall olfactory response.

Preliminary Biosynthesis Experiments

Tracer experiments were carried out a) to determine whether danaid butterflies could synthesize their heterocyclic pheromone from simple compounds which might serve as plausible endogenous precursors and b) to see the effect of injection of potential exogenous precursors.

a) *Danaus gilippus berenice* Cr. males captured in the field (Hendry County, Florida) were used for these studies. The isotopically labelled potential precursors were injected, and subsequently the pheromone (I) was isolated, purified, and assayed for radioactivity. Using sodium acetate (2-^{14}C), sodium mevalonate (2-^{14}C), proline (5-^{14}C), ornithine (5-^{14}C), and δ-aminolevulinic acid (4-^{14}C), no incorporation of radioactivity was observed (for experimental details see Thompson unpubl.).

b) Pliske (pers. comm.) observed that *D. gilippus* males congregate on *Crotalaria spectabilis* Roth. (Leguminosae). This plant is known to contain the pyrrolizidine alkaloid monocrotaline (Leonard, 1950/60). Retronecine (III) (Fig. 4), the heterocyclic moiety of monocrotaline, was obtained by alkaline hydrolysis of a sample of this alkaloid which was isolated from *C. spectabilis*. Oxidation of III to the corresponding methyl ester followed by lithium aluminium tritiide reduction, as outlined in Fig. 4, gave specifically labelled base (III), which was then acetylated to give the diacetate (IV).

A small group (5) of *D. gilippus* males were injected with IV dissolved in insect Ringer solution, and kept in glassine envelopes. They were fed to satiety daily on aqueous sucrose-honey solution. After six days, the hairpencils were removed and extracted with methylene chloride. A small amount of cold carrier (I) was added to the extract, and the isolated I was purified to constant radioactivity by five-fold recrystallization of its oxime from 50% aqueous ethanol. The

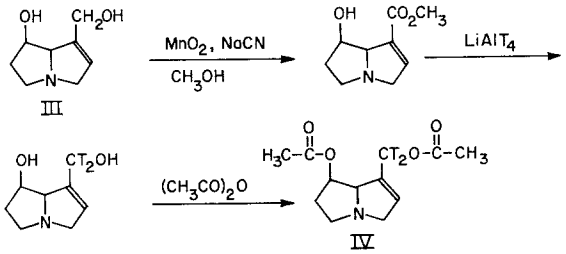


Fig. 4. Preparation of specifically labelled retronecine diacetate (*IV*)

incorporation of radioactivity from the alkaloid derivative (IV) into the pheromone (I) amounted to a low, but significant 0.015%.

While these exploratory experiments cannot be taken in themselves to indicate the natural biosynthetic pathway to I in *D. gilippus*, they are in accord with the hypothesis of a necessary alkaloid precursor, as supported by the other results included in this report.

Discussion

Pheromones are signal compounds which serve for intraspecific chemical communication. In general, little is known about their biosynthesis (see Karlson and Schneider, 1973). The question of whether a particular pheromone can be "built" from metabolites which are available as a result of the catabolic breakdown of the normal food, or whether a special food is required, has rarely been raised. In the case of the Gypsy Moth, it was found that the female moth emitted a reduced amount of its pheromone when the larvae were raised in the laboratory (Richerson and Cameron, 1974).

In Lepidoptera, the basic building stones for the development of the adult insects are ingested by the larvae. In the majority of species the adults (if they feed at all which is not the case in a number of moths) obtain carbohydrates from nectar to serve their energy requirements. There exist, however, quite interesting specializations as to what food source a given species prefers to visit. Among the butterflies, it is well known that some groups (often only males) are strongly attracted by fermenting fruits (Nymphalidae), by feces (*Charaxes*, *Apatura*, *Limenitis*), by decaying meat (Papilionidae, Pieridae, Lycaenidae), or by mammalian urine (several genera) (Owen, 1971; Lederer, 1951). Furthermore, one knows of the attractiveness of puddles to butterflies; Arms *et al.* (1974) recently reported that male *Papilio glaucus* L. (Papilionidae) prefer food sources which contain NaCl. We know virtually nothing, however, about the biological or biochemical meaning of these preferences.

In Danaidae, males are attracted to and suck on certain plants. Fortunately for our consideration, those plants (see Introduction) have been rather extensively analyzed chemically for their toxins (hepatotoxic pyrrolizidine alkaloids), which not only are a risk for livestock, but are also interesting for their pharmacological and pharmaceutical properties.

Interestingly, the male hairpencils of 17 danaid species have been found to contain between one and three related heterocycles (substance I, and two related aldehydes; see Schneider, 1974/75) which were observed to serve (or very probably serve) as male sexual pheromones. The widespread occurrence of substance I or related compounds in the Danaidae was predicted after EAG cross-tests with the hairpencils of seven species (Schneider, 1971). So far, pyrrolizidines seem to be absent in only four species of Danaidae: *Danaus plexippus* L., *D. formosa* Godman, *Amauris oscarus* Thurau, *Euploea core* Cramer, and *E. boisduvali* Lucas (Meinwald *et al.*, 1971; Edgar *et al.*, 1971, 1973; Meinwald *et al.*, 1974; Schneider, 1974/75). However, EAG tests with the first three of these species revealed that they do possess odor receptors for substance I. Whether the lack of heterocyclic pheromones in some or all of these species is due to a (possibly even seasonal) reduced or blocked access to the plants which contain the pheromone precursor, is the subject of work in progress. At least in *D. plexippus* (the American Monarch), which is a long-lived migratory butterfly in large parts of the world, pyrrolizidines were found missing in specimens from North America, the New Hebrides and New South Wales (Meinwald *et al.*, 1971; Edgar *et al.*, 1973).

Based on the observations that (1) pyrrolizidine alkaloids occur in plants frequented by male danaids, (2) pyrrolizidines occur in hairpencils of field-caught danaids, and (3) no pyrrolizidines were found in hairpencils of indoors-raised danaids, the following working hypothesis was proposed. Male danaids are attracted to and feed on plants which contain pyrrolizidine alkaloids; these compounds are an essential dietary factor without which production of the pheromone is impossible. Evidence in favor of this hypothesis was presented for the Australian *D. chrysippus petilea* (Edgar *et al.*, 1973). The results of our present experiments are in full agreement with the working hypothesis as outlined in the introduction.

Our recent findings, which indicate that one single EAG exposure of one hairpencil of a pair leads to a reduction of its pheromone content by almost 50% compared to the hairpencil which was not exposed (Table 1, lines 4 and 5), are not in agreement with our earlier findings that hairpencils (of field-caught animals) are effective odor sources for months or even years when kept at 4–5° C in between use (Schneider and Seibt, 1969). One possible reason for this discrepancy might be that with the first EAG stimulus, much of substance I is lost with the dust blown from the hairpencil. In addition, evidence is accumulating that the danaid hairpencils contain a number of compounds other than the pyrrolizidines, of which at least some could be rather effective EAG stimuli. EAG cross-tests with hairpencils of *Danaus formosa* and *Amauris oscarus*, which lacked the pyrrolizidines, elicited large EAGs even in species which normally contain them (Schneider, Boppré and Eisner, unpubl. results). The chemotactical orientation of male *D. chrysippus* to withered but moistened *Heliotropium* corresponds well to the response of antennal odor receptors when stimulated with air blown over such plants (Fig. 3). The olfactory stimulus could in these cases easily be the alkaloid (II) (which in fact also elicits an EAG). However, it must remain an open question whether this large alkaloid molecule, or only its heterocyclic moiety after hydrolysis, acts as the olfactory principle to lure the animal to the plants. In this context, Edgar *et al.* (1973) not only found that withered *Helio-*

Table 2. Interrelationship between Danaidae and poisonous plants

	Sub-families	Plants	Toxins
larval food plants	Lycoreinae	Asclepiadaceae	cardiac-glycosides
	Euploeinae	Apocynaceae	alkaloids, glucosides
	Danainae	Asclepiadaceae	cardiac-glycosides
♂ (♀) imago attractant	Danaus chrysippus D. gilippus ? D. limniace ? Amauris ochlea ? A. niavius ?	Heliotropium (Boraginaceae) Senecio ? (Compositae) Crotalaria ? (Leguminosae)	pyrrolizidine alkaloids

tropium needs to be moistened ("activated") before it becomes attractive, but also report that chloroform washings of moistened, but never of dry, *Heliotropium* contained a pyrrolizidine hydroxy aldehyde.

The butterfly family Danaidae provides a fascinating example of a close interrelationship between insects and flowering plants. It has been known for some time that danaids depend on heart toxins (cardenolides, see Reichstein *et al.*, 1968), found in the plant families Asclepiadaceae and Apocynaceae, for their protection. Because of the presence of these compounds in the larval food, larvae and adults are unpalatable to their bird predators (Brower, 1969). This unpalatability of the danaids is then "used" by butterflies (and possibly even some moths, e.g. the partly diurnal geometrid *Aletis helcita*) of several families which mimic the danaid wing pattern. As a result of the present work on *Danaus chrysippus*, it is apparent that this species (and very likely also *Danaus gilippus berenice*) needs to acquire the alkaloid precursor of its male pheromone from plants. The surprising fact is that it is the *adult* male butterfly which actively searches for these compounds in the plants; the larval foodplant does not contain the necessary precursors (Edgar *et al.*, 1973). The resultant complex set of interrelationships of danaid butterflies with poisonous plant families is summarized in Table 2.

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