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Behaviorally Mediated Contacts Between Scent Organs: Another Prerequisite for Pheromone Production in *Danaus chrysippus* Males (Lepidoptera)

Michael Boppré [1], Robert L. Petty [2], Dietrich Schneider [1], and Jerrold Meinwald [2]

[1] Max-Planck-Institut für Verhaltensphysiologie, D-8131 Seewiesen, Federal Republic of Germany
[2] Department of Chemistry, Cornell University, Ithaca, NY 14853, USA

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Summary. Males of *Danaus* butterflies possess two binate glandular organs: abdominal hairpencils, which release aphrodisiac pheromones during courtship behavior, and pockets on the hindwings. Between these two types of organs contacts are established behaviorally: the hairpencils are dipped into the alar pockets (Fig. 2). GLC analyses of hairpencil and wing pocket extracts from *Danaus chrysippus* show that this contact behavior is a prerequisite for the synthesis of the ketonic pheromone component (for which the term "danaidone" is suggested here) in physiologically normal amounts. Danaidone occurs on the hairpencils only i) after the males have ingested pyrrolizidine alkaloids as precursors, and ii) after the hairpencils have been dipped into the wing pockets. The appearance of danaidone in the wing pockets also depends on the ingestion of alkaloidal precursor, but is not affected by the above mentioned contact behavior. Mechanisms by which contact behavior might control pheromone biosynthesis are discussed.

Introduction

During courtship flight, male danaid butterflies protrude and expand abdominal hairbrushes in front of their females (Brower et al., 1965; Seibt et al., 1972). These "hairpencils" contain aphrodisiac pheromones which stimulate females for mating (Pliske and Eisner, 1969; Myers and Brower, 1969). Three prominent volatile and biologically active components of the hairpencils scent in many danaids have been characterized as dihydropyrrolizines (Meinwald et al., 1966; Edgar et al., 1973; see Schneider, 1975, and Edgar, 1975, for ref.).

The biological importance of these dihydropyrrolizines in Danaidae leads us to suggest the following trivial names: 1. *danaidone* for the ketone, 6,7-dihydro-1-methyl-5H-pyrrolizine-7-one (Fig. 1A), found in many species of the genera *Danaus* and *Amauris*, 2. *danaidal* and 3. *hydroxy-danaidal* for 6,7-dihydro-5H-pyrrolizine-1-carboxaldehyde (Fig. 1B) and 7-hydroxy-6,7-dihydro-5H-pyrrolizine-1-carboxaldehyde (Fig. 1C) respectively, which are common in the genus *Euploea*.

Danaidone has been shown to serve as an aphrodisiac pheromone necessary for successful courtship in *Danaus gilippus berenice* (Pliske and Eisner, 1969) and in *D. chrysippus* (Boppré and Schneider, unpubl.). For other species possessing danaidone or the danaidals, the same function can be anticipated, although additional functions are also possible.

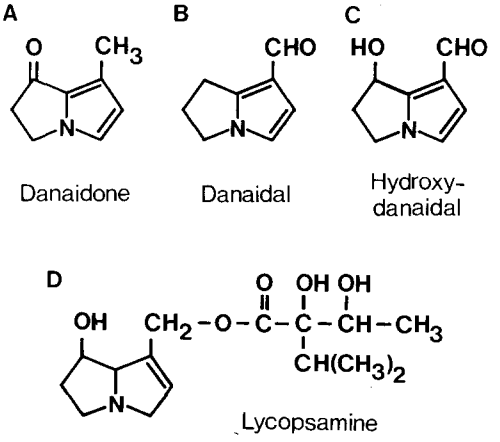

Fig. 1A–D. Dihydropyrrolizines (**A**, **B**, **C**) found on hairpencils of various danaid butterfly species, and the pyrrolizidine alkaloid lycopsamine (**D**)

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Besides the pair of glandular hairpencils at the tip of the abdomen, males of the majority of species in the subfamily Danainae possess a patch or a pocket with secretory cells on each hindwing (for morphological details and references see Brower et al., 1965; Boppré et al., 1978). When F. Müller described these organs in *Danaus chrysippus* for the first time over a hundred years ago, he suggested that there might be interactions between the hairpencils and the wing pockets because of their respective position and shape "... it would not be impossible or even difficult to introduce them (the hairpencils) into the depth of the cavity" (Müller, 1877).

Subsequently, contacts between abdominal and alar scent organs have indeed been observed in a number of species. Independent of and prior to courtship behavior, male *Danaus* insert their hairpencils into the wing pockets, and male *Amauris* apply the outer part of their complex hairpencils to the wing patches (see Brower and Jones, 1965, for ref.; Seibt et al., 1972; Boppré, 1977).

Concerning the function of the alar glands and the significance of contacts between the hairpencils and these organs, a variety of hypotheses have been formulated. However, none of the experimental investigations described so far (Brower and Jones, 1965; Pliske and Eisner, 1969; Seibt et al., 1972; Myers, 1972) explained the meaning of this behavior (see Discussion).

The finding that the biosynthesis of danaidone is dependent on the ingestion by the males of pyrrolizidine alkaloids (Edgar et al., 1973; Schneider et al., 1975; see also Edgar, 1975) has led us to examine whether contacts between abdominal and alar organs in *Danaus* are an additional prerequisite for danaidone biosynthesis.

Material and Methods

Our experiments were carried out with indoor-raised males of *Danaus chrysippus f. chrysippus* L. and *D. chrysippus f. dorippus* Klug. The butterflies were reared from eggs (of females caught in Kenya or mated in the greenhouse) on *Asclepias curassavica* L. and *Gomphocarpus physocarpus* E. Mey. (Asclepiadaceae) in greenhouses in Seewiesen.

The butterflies were kept in groups of ten, either in insect cages ($42 \times 42 \times 42$ cm) exposed to direct sunshine in the greenhouse, or else free in the greenhouse. After feeding the males for 6 to 18 days, extracts of hairpencils and wing pockets were analyzed with a gas chromatograph.

Males were individually marked and subjected to experiments in which i) their ability to ingest pyrrolizine alkaloids, and ii) their ability to carry out the above-described contact behavior was independently varied (cf. Table 1).

(i) Males were given access either to sugar/honey water solution alone or – in addition – to withered plant material of *Heliotropium steudneri* Vadtke (Boraginaceae), which contains the pyrrolizidine alkaloid lycopsamine (Fig. 1 D) (Schneider et al., 1975).

(ii) While contacts between hairpencils and wing pockets were allowed in some of the butterflies (groups A, C, D), contacts were mechanically hindered in others (groups B, E). In the latter groups prior to any contact (i.e. immediately after the wings were hardened after eclosion) pockets were either cut out or their openings were closed with small lamina of aluminium foil which were fixed with droplets of low melting point wax. In order to exclude the influence of individual variation due to differences in the precursor uptake, contacts were mechanically hindered on one side of the body only in a number of specimens (group F). In these cases, any chemical difference between the two hairpencils must be the result of contacts and not of the precursor uptake.

For chemical investigation, the glands were stored in CS_2. In preliminary analyses, carried out in Ithaca, extracts of glands of 1 to 5 individuals were combined and analyzed. The CS_2 solution in which the glands were stored (<0.5 ml) was removed and the glands were rinsed with CS_2. The solutions were combined and the resulting extract was concentrated to a known volume. (This single rinse method was shown to remove about 80% of the extractable material.) A portion of the extract was then chromatographed on a $8' \times 2$ mm glass column packed with 2% OV-17 on 100/120 mesh Gas Chrom Q, in a Varian 2100 gas chromatograph utilizing a flame ionization detector (FID). The temperature was programmed from 102° to 150° at 4 °C/min. The identification of danaidone was confirmed in selected samples by direct comparison with an authentic sample *via* combined gas chromatography/mass spectroscopy, using a computerized Finnigan 3300 GC/MS.

The final data, reported here in detail, were obtained from single hairpencils and wing pockets or from pairs of these glands belonging to individual specimens, analyzed in Seewiesen. The organs were extracted with 50 or 100 µl of CS_2, 5 or 10 µl of this solution being used for direct injection into a Hewlett Packard 5830 GC which was equipped with a $8' \times 2$ mm glass column filled with 2% OV-17 or OV-1 on 100/120 mesh Gas Chrom Q and a FID. The temperature was kept constant at 150° or 170 °C. Danaidone identity was confirmed by coinjection with the synthetic compound; absolute amounts of danaidone on hairpencils and in wing pockets were calculated by comparison of GLC peak areas recorded after injection of extracts with peak areas produced by known amounts of synthetic danaidone. The detection limit was 0.05 µg.

Finally, using the latter method, we analyzed the hairpencils and wing pockets of a number of individual males which were caught in the field in Kenya, in order to assess the physiologically normal amounts of danaidone and its variation between individuals (group G).

Results

A. Behavior

Our observations of dipping the hairpencils into the alar pockets ("contact behavior") confirm the information reported by Brower and Jones (1965) for *Danaus gilippus xanthippus* Felder. In *Danaus chrysippus*, contact behavior was observed at any of time of the day. However, the butterflies prefer sunny morning hours; they alight on exposed locations, usually on those which receive direct sunshine. Prior to insertion of the hairpencils into the wing pockets, the males show turning movements of the head, twitching of the terminal segments of the abdomen, and they

close their wings and lift up their forewings. The abdomen is then bent dorsally so that its tip approaches the openings of the pockets, and the two hairpencils are pushed synchronously into the cavities of the two hindwing pockets and held there for a few seconds (Fig. 2). Inside the small pockets, the hairpencils cannot be expanded to spheres as they are during courtship behavior; in fact they stay as bundles even if the wing pockets have been cut out.

Fig. 2. Male of *Danaus chrysippus* attempting to introduce its hairpencils into the wing pockets. One hairpencil is seen protruding through a small hole cut into the underside of the right wing pocket

Air-dried plant material of *Heliotropium steudneri* was a powerful attractant for the males, which were able to locate the plants in an upwind flight. After probing the surface of the plants with the antennae and the proboscis, the butterflies use their proboscis to apply droplets of a fluid which they then reimbibe. Sucking at one spot for more than half an hour was not unusual.

B. Chemistry

The amounts of danaidone detected by gas chromatography in extracts of hairpencils and wing pockets of experimental males are listed in Table 1.

For the appearance of *danaidone on the hairpencils* these data show:

1. Contact behavior has no effect unless the males have had access to pyrrolizidine alkaloids (group C).
2. Following the uptake of pyrrolizidine alkaloids, the hairpencils contain danaidone only if contacts have been established (cf. groups A and D with B and E).

Table 1. Amounts of danaidone detected by GLC analyses in extracts of hairpencils and wing pockets of males which varied with respect to i) access to pyrrolizidine alkaloids and ii) contacts between abdominal and alar glands. Detection limit: 0.05 µg. The values for groups A and B originate from combined extracts of 5 (3) males, those for groups C, D, E, and G from extracts of pairs of hairpencils or wing pockets of individual males. In group F, individual scent organs rather than pairs were analysed; the measured amounts are doubled in the Table in order to facilitate the comparison with other groups

Group	No. of males	Food	Contacts between hairpencils and wing pockets?	Amount of danaidone (µg/male)			
				On hairpencils		In wing pockets	
				Range	Average	Range	Average
A	5	Sugar/honey water and access to withered *Heliotropium* plants	Yes	–	**145**	–	**9**
B	3		No	–	**0**	–	**9**
C	10	Sugar/Honey water only	Yes	–	**0**[a]	–	**0**[a]
D	11	Sugar/Honey water and access to withered *Heliotropium* plants	Yes	16 –163	**84**	2.1–13	**6.1**
E	10		No	–	**0**[a]	0.8– 5.1	**4.5**
F	8		Yes	14 –303	**123**	2.6–11	**5.2**
			No	0.6–2.3[b]	**< 1**[b]	–	–
G	19	(Probably nectar and pyrrolizidine alkaloids)	(Probably yes)	31 –420	**212**	0.9–11	**3.6**

[a] Small danaidone peaks, amounting to less than 0.6 µg per insect, were observed in chromatograms of four individuals. These traces are most likely due to alkaloid contamination occurring in insect cages or to danaidone contamination during sample preparation

[b] Traces of danaidone on these uncontacted hairpencils obviously originate from the contacted hairpencils; due to the location of the hairpencils in the abdomen, the two organs come into contact when protruded

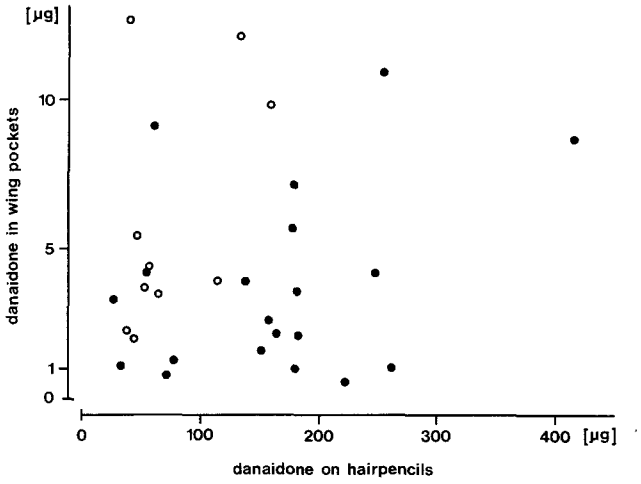

Fig. 3. Danaidone content of hairpencils compared with that of wing pockets of 30 individual *D. chrysippus* males. ● field-caught males (group G); o experimental males (group D). (Note the different scales on the axes!)

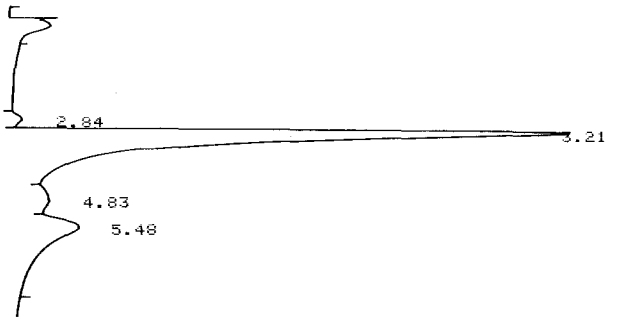

Fig. 4. Typical gas chromatogram of a hairpencil extract of *D. chrysippus* (OV-1 column, 150 °C). Numbers indicate retention times in min. Reproduction of original recording

3. The possibility that this lack of danaidone on hairpencils in the absence of contacts (see 2.) is due to some factor other than the contact itself (such as insufficient uptake of pyrrolizidine alkaloids) is ruled out by the group F experiments: only the hairpencils on the side of the body where contacts were permitted contained danaidone.

4. The amount of danaidone found in individual males varies considerably (groups D, E, F).

The appearance of *danaidone in the wing pockets* also requires ingestion of pyrrolizidine alkaloids (cf. group C with all others). However, danaidone is found in wing pockets independent of contacts with hairpencils, although always in comparatively small amounts.

In *field-caught males* (which have probably ingested alkaloids and have contacted the glandular organs) the hairpencils contain more danaidone on the average than those of comparable experimental males (cf. group G *vs.* A, D, F), but the amounts of danaidone still vary over a wide range. Wing pockets of field-caught males again possess only small amounts of danaidone, as was the case for the experimental males.

While Table 1 lists averaged data, Figure 3 gives some representative analyses of danaidone amounts found in individual males. These data demonstrate that there is no correlation between the amounts of danaidone found on hairpencils and in wing pockets, i.e. a male with a large quantity of danaidone on its abdominal scent organs has neither an especially large nor an especially small amount of danaidone in its wing pockets.

The gas chromatogram of hairpencil extracts (Fig. 4) shows one major peak (danaidone, retention time = 3.21 min) and three minor peaks. One of the small peaks is due to a terpenoid diol (*E*-3,7-dimethyloct-2-en-1,8-diol, rt = 5.48; Meinwald et al., 1971); the other two are unidentified. Wing pocket GLCs – independent of contacts – show only the danaidone peak.

Discussion

Several hypotheses have been put forward in the literature to explain the function(s) of the wing pockets, as well as of the contacts between hairpencils and wing pockets in male danaid butterflies. The initial ones are given below (cf. reviews by Brower et al., 1965, and Brower and Jones, 1965):

(i) The wing pockets produce some odoriferous substance and the hairpencils disseminate it (Müller, 1877).

(ii) The wing glands and the abdominal glands might produce two different volatile substances which, when mixed, produce the characteristic perfume of the species (Eltringham, 1929; Latter and Eltringham, 1935).

Brower and Jones (1965) made the first experimental attempts to clarify the meaning of contact behavior. In their studies with *Danaus gilippus*, these authors sealed the wing pockets chiefly of field-caught males and tested – before and after contacts had been hindered for several days – the odor of both organs as perceived by the human nose. Their results are equivocal, however, because of the methods used. The human nose (aside from the problems of subjective sensation) is not necessarily a detector for biologically meaningful compounds. Danaidone itself, for example, is odorless for humans. In addition, it is most likely that field-caught males have already

performed contact behavior. Furthermore, from our experiments with *D. chrysippus* and other species, the hairpencil odor stems from compounds which are still unknown, and varies in a way which does not seem to be correlated with contact behavior.

Thus, for testing the function(s) of contacts between the two glands in danaids experimentally, it is necessary i) to work with freshly emerged butterflies (in order to be able to control the occurrence of contacts), and ii) to do comparative studies on contacted and uncontacted organs using techniques which allow a biologically relevant interpretation, i.e. studying behavior or analyzing chemicals with known functions.

Experiments carried out by Pliske and Eisner (1969) (cf. Pliske, 1968) met these requirements. They "removed" the wing pockets of freshly emerged *D. gilippus* males and found that the wing glands affected neither the occurrence of danaidone nor courtship behavior and sexual potency. These data disagree with our present findings, but the discrepancy can be explained. Pliske (1968) peeled the structure off the upper lamella of the wings with forceps; we believe that with this technique the glands were only incompletely removed, and could still perform their function.

Seibt et al. (1972) tried to relate contact behavior to danaidone deficiency in indoor-raised *D. chrysippus* males. They carried out experiments which were monitored by electrophysiological tests as well as by thin-layer chromatography. But since the necessity for an alkaloidal precursor was not yet established, they were unable to observe any significant effects.

The present results confirm that alkaloid uptake is an indispensable first requirement for danaidone biosynthesis. Additionally, the experiments demonstrate that behavioral contacts between hairpencils and wing pockets are a second indispensable requirement for the appearance of danaidone on the hairpencils. The wing pockets, on the other hand, always contain small amounts of danaidone after ingestion of pyrrolizidine alkaloids independent of contacts. This means that a male can synthesize physiologically normal amounts of danaidone (i.e. comparable to those in field-caught males) only if it is able to bring its hairpencils into contact with its wing pockets.

Our chemical analyses of scent organs of individual males allow a more accurate description of the amounts of danaidone in *Danaus chrysippus* than could be given from previous studies of pooled extracts of several organs. Individual hairpencils of field-caught males from Kenya contained up to 400 µg/pair of hairpencils (see Table 1, group F). This is over twice as much as the previously reported average danaidone content of hairpencils of butterflies collected in Sierra Leone (Meinwald et al., 1971). The wide individual variation in danaidone content is not surprising, since both an ecological and a behavioral factor are involved in its biosynthesis. In addition, the field-caught animals were unavoidably of different ages and might have lost part of their danaidone because they disseminate pheromone-transfer-particles impregnated with danaidone in the course of courting their females (Boppré et al., 1978).

The fact that the maximal amount of danaidone on the hairpencils of the experimental males was lower than the maximum found in field-caught males is most probably due to the less than optimal laboratory conditions:

1. Since the alkaloid content of plant material varies (as known from analyses of plant extracts), the plant material presented to the experimental males may have been the limiting factor.

2. The conditions which promote contact behavior were probably not as good in the laboratory as in the field (light, temperature, humidity, etc.).

So far, we have only given a description of the results which indicate that hairpencil/wing pocket contacts are necessary for a normal level of danaidone biosynthesis. While the detailed pathway for this biosynthesis is still unknown, we can point out two mechanisms (A and B) which would be in agreement with our data (cf. Fig. 5). Both possibilities are based on the assumption that the wing pockets contain enzymes which convert pyrrolizidine alkaloids into danaidone.

A) The ingested alkaloid is transported to the wing pockets where it is enzymatically converted into danaidone; from the pockets, danaidone is picked up and accumulated by the hairpencils.

B) Only a small fraction of the ingested alkaloid reaches the wing pockets and is converted into danaidone. However, most of the alkaloid accumulates in the hairpencils and is converted into danaidone on a larger scale as a result of the contact behavior, which brings together enzymes from the pockets and alkaloid from the hairpencils.

The first mechanism might imply that the wing pockets are a reservoir for danaidone and (unless danaidone biosynthesis is inhibited by the accumulation of the product) it might be expected to result in large amounts of danaidone in the wing pockets, especially in those cases where these had no contact with the hairpencils. The fact that this implication is not realized together with the observation of Edgar and Culvenor (1974), who found unconverted alkaloids in extracts of hairpencils, favors the second mechanism over the first. In any case, it is too early to reach any definite conclusions, and we hope that studies now in progress will give more insight into this biosynthetic mechanism.

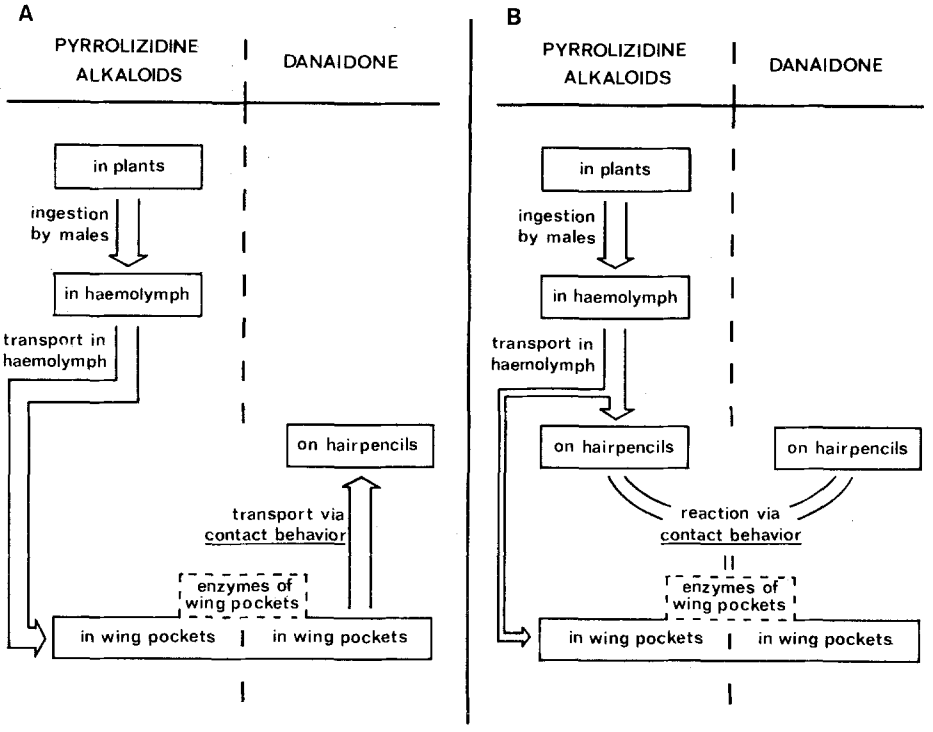

Fig. 5A and B. Two mechanisms which could explain the observed effects of contact behavior on danaidone biosynthesis

Even now, however, the information available demonstrates the striking complexity of pheromone biology in danaid butterflies (cf. Boppré, 1977, 1978). Before mating, male *Danaus chrysippus* (and probably other species as well) have to search for withered, pyrrolizidine alkaloid-containing plants. They then have to dissolve and take up these precursors. Finally they have to bring about contacts between their abdominal hairpencils and their alar glands in order to produce danaidone, which is an essential stimulus to the female during courtship.

Why two glandular systems and a special behavior pattern are required for danaidone biosynthesis remains a puzzle, although multicomponent scent organ systems are known in several other butterflies and moths (see e.g. Clearwater, 1975a). For the noctuid *Mamestra configurata* Walk., more than a morphological description of the organs is available (see Clearwater, 1975b, and ref. therein): one gland produces the pheromone precursor (β-phenylethyl glycoside), which is conducted to the glandular hairpencils. The basal cells of the hairpencils (presumably) then supply the enzyme (β-glycosidase) necessary for the conversion of the precursor into the aphrodisiac pheromone (phenylethanol). In this case, the interaction of the products of two glands is a prerequisite for pheromone biosynthesis, but – in contrast to the danaid case – contacts between these glands are not established behaviorally. The interpretation that the noctuid moth needs the two glands because the pheromone itself is detrimental to the cells does not seem applicable to the danaids. Here, even though the alkaloidal precursor is very toxic, the butterflies tolerate and even store the alkaloids, presumably for defensive purposes (Edgar et al., 1976; Edgar et al., 1978.

Other danaid species also produce danaidone and are attracted to pyrrolizidine alkaloid-containing plants; it was found that the dependency on pyrrolizidine alkaloids is not exceptional in *Danaus chrysippus* (unpubl. data of the present authors; see also Edgar, 1976). We also have evidence suggesting that in other danaid species contact behavior is necessary for pheromone production. Nevertheless, in the danaid *Lycorea ceres ceres* Cramer no alar glands are known. However, males of this species are attracted to and suck on alkaloid-containing plants (Beebe, 1955), and they too produce danaidone (Meinwald et al., 1966). It follows that there is significant variation in the details of how danaids accomplish danaidone biosynthesis.

Thus far, we have discussed the wing pockets in danaids only in connection with danaidone biosynthesis. Nevertheless, these glands could very well have functions other than or in addition to that of danaidone production. Contacts between abdominal and alar glands in the family Danaidae may have other or additional functions too. In *Danaus limniace pe-*

tiverana Doubleday and Hewitson, for example, contact behavior causes specific morphological effects. In this (and some other) species, pheromone-transfer-particles are produced inside the alar pockets rather than on the hairpencils as in *D. chrysippus*. By means of contact behavior, these particles are taken up by the hairpencils, from which they are disseminated during courtship (Boppré and Fecher, 1977; Boppré et al., 1978). Accompanying chemical effects are also quite likely, however, and these phenomena are now under investigation.

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