Identification and biological significance of 4-methyl-3Z,5-hexadienoic acid produced by males of the gall-forming tephritids *Urophora cardui* (L.) and *Urophora stylata* (Fab.) (Diptera: Tephritidae)

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Received 8 August 1989; accepted 25 October 1989

Summary. A new natural substance has been identified in the rectal ampullae of gall-forming fruit flies. The substance was found to be the only volatile compound in the rectal ampulla of male *Urophora cardui* and *Urophora stylata*. GC-MS methods were used to characterize its structure as 4-methyl-3Z,5-hexadienoic acid. Physiological parameters such as the amount of the acid at different ages and under different conditions were investigated. The biological significance of the new volatile as an arresting pheromone was tested in several bioassays. The arrestant function could not be established, but the results gave hints of a territorial function between conspecific males. The results are discussed with respect to gland morphology and predictions of communication models among fruit flies.
Key words. Tephritidae; *Urophora cardui* (L.); *Urophora stylata* (Fab.); volatile; 4-methyl-3Z,5-hexadienoic acid; gas chromatography; mass spectrometry; quantitative analysis; bioassay.

Today many volatile substances are known to be produced by tephritids. In several cases they have been proved to act as aggregation or sexual pheromones. All of these investigations were conducted among the ecological group of tropical and subtropical tephritid species, the typical fruit flies, since these are often serious crop pests. Most of the species examined belong to the subfamily of the fruit-infesting Dacinae. The subfamily Trypetinae includes both fruit-infesting and gall-forming species. The genus *Urophora* (Trypetinae) is a monophagous gall-forming tephritid of temperate regions. The biology of this phytophagous insect and its interaction with its host plant were studied by Zwölfer[1] in detail. Many species of this genus are of importance with respect to the biological control of weeds, especially thistles, because they can reduce the reproductive capacity of their host plants by concentrating nutrients and energy in a new plant organ, the gall, which is induced by the larvae. The application of *Urophora cardui* as agents in the biocontrol of *Cirsium arvense* (L.) Scop. in Canada was attempted, but led to problems, because the flies were not dispersing fast enough to be effective agents.

A semi-field test in Canada (Peschken 1982, unpublished) gave reason to suggest a preferred oviposition in stems of thistles that were marked by males. Harris[2] described a strong odor which was emitted by male *Urophora cardui, U. stylata, U. affinis* (Frauenfeld) and *U. quadrifasciata* (Meig.). In the case of *U. cardui* the odor was strong enough to be detected in the field. Harris supposed that it might be an arresting pheromone. These and our own observations led Zwölfer (1987, pers. comm.) to the hypothesis that the volatile might act as an arrestant for the vagile females of *U. cardui*, to ensure the encounter of sexual partners on their host plant. To test this hypothesis we worked on different topics. The site of synthesis and storage in the flies was determined. For identification, the substance was isolated and the natural product was compared with different synthetic products. The quantitative analysis of *Urophora cardui* and *U. stylata* with respect to age and several bioassays gave hints as to biological function.

Material and methods

Fruit fly material. Galls of *Cirsium arvense* containing *U. cardui* larvae were collected in early spring 1988 near Grafenwöhr (Oberpfalz), and *Cirsium vulgare* (Savi) Ten. galls with larvae of *U. stylata* in autumn 1987 near Kassel (Nordhessen). After three weeks at 21 °C in the laboratory the adult flies emerged. They were kept under constant conditions in petri dishes singly, pairwise or in groups of three individuals. To establish the influence of nutrition on the synthesis of the volatile, some of the flies remained unfed while the others had access to an aqueous solution of honey. In total 134 males/14 females of *U. cardui* and 73 males/4 females of *U. stylata* were analyzed quantitatively.

Collection of volatile. In order to get the total amount of volatile from one individual it was necessary to dissect each fly and to prepare the rectal ampulla, which is a part of the hindgut and serves as a reservoir for the volatile. The whole content of the rectal ampulla was taken without solvent into a glass capillary and stored at − 30 °C.

Identification by high resolution mass spectrometry. The molecular mass of the volatile was determined by high resolution measurements (peak matching; PFK-samples) using a Varian MAT 311 A mass spectrometer. Whole rectal ampullae were fractionated and vaporized to record all volatile substances.

Derivatization. The content of 1–5 male rectal ampullae was esterified with diazomethane. Double bonds of the esterified natural substance were hydrogenated with PtO_2/H_2, and the mass increment in GC-MS analysis noted.

GC-MS. The GC-MS analysis was done with a Finnigan/MAT Iontrap ITD 800 (70 eV) combined with a Carlo

Erba Vega 6000 capillary gas chromatograph. Separation of compounds was achieved by using a CW 20M Permabound fused silica capillary column (25 m * 0.32 mm ID * 0.25 µm) under programmed conditions from 55 °C (2 min) to 220 °C at 15 °C/min.

GC-FTIR. Infrared spectra were recorded on a Bruker IFS 48 GC/FTIR instrument, combined with a Carlo Erba gas chromatograph, model Mega HRGC 5300. Compounds were separated on a BP 1 capillary (25 m * 0.32 mm) under programmed conditions (70 °C for 3 min, followed by 10 °C/min to 200 °C). The light pipe was kept isothermal at 250 °C. A sampling rate of 8 scans/s, averaged to a single scan, was used.

Quantitative gas chromatography. This analysis was done by a Carlo Erba Mega HRGC 5160 gas chromatograph, equipped with a FFAP capillary column for free fatty acid separation (10 m * 0.32 mm ID * 0.25 µm) under programmed conditions (85 °C for 1 min, followed 10 °C/min to 220 °C), using an on-column injection technique and FID-detection. Helium was used as carrier gas (40 kpa; 2.4 ml/min at 85 °C). The rectal ampulla of each fly was dissected and its secretion completely transferred to a 1-µl micropipette. A definite volume of benzene was used as solvent, containing caproic acid as internal standard. Immediately after this preparation procedure, each sample was analysed by gas chromatography. The calculation of the peak areas was done by a Spectra Physics Integrator (System 1).

Bioassays. According to the hypothesis of an arrestant pheromone we expected the females to be attracted by released rectal ampulla content of males and to oviposit preferably in host plants marked by the volatile. All experiments were conducted with males and females of *Urophora cardui.* In a first approach we tried to repeat the results of the experiment of Peschken (1982, unpublished) with host plants which were marked for 3 days by caged males. Subsequently the males were removed and females were put in the cages. We noted their residence on marked and unmarked plants and counted the galls on each plant 6 weeks later. For a standardized choice test in the lab, a vertical glass Y was used. In each test a group of 10–15 flies was put one after another on the lower half of the Y; from there they started to walk upwards and went to the right or the left arm of the Y. The glass Y was marked by a small ring of rectal ampulla content from one *U. cardui*-male around one arm, near to its base. Comparing tests and controls we could note differences from the normal distribution observed in control tests.

The potential repellent effect of the rectal ampulla content was investigated on ants and spiders in separate experiments.

Results

Identification and synthesis of 4-methyl-3Z,5-hexadienoic acid. Gas chromatographic and mass spectroscopic analysis of the highly volatile and strongly odoriferous substance (reminiscent of phenylacetic acid) from the rectal ampulla revealed only a single compound. According to HR-MS the elemental composition of its molecular ion (126.148 Da) is $C_7H_{10}O_2$, suggesting the presence of an unsaturated acid. This is readily confirmed by esterification with diazomethane ($M^{+\cdot} = 140$ Da) and hydrogenation (PtO_2/H_2), which accounts for two double bonds ($M^{+\cdot} + 1 = 145$ Da). An intense fragment of the saturated ester at m/z = 74 (fig. 1) results from β-cleavage and hydrogen rearrangement (McLafferty product) and is indicative for an unsubstituted polar head (C(1)-C(3)). Since the compound is not identical with methyl heptanoate, a branch has to be present at the aliphatic terminus. Further information is accessible from GC-FTIR spectra of the esterified natural product (fig. 2). The strong absorption at 1763 cm^{-1} is due to a nonconjugated carbonyl group, and the two weak bands at 1018 and 914 cm^{-1} are accounted for by an unsubstituted

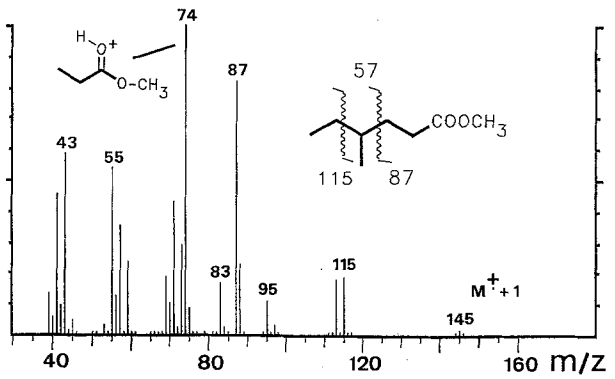


Figure 1. EI-MS spectrum of hydrogenated and esterified authentic 4-methyl-3Z,5-hexadienoic acid.

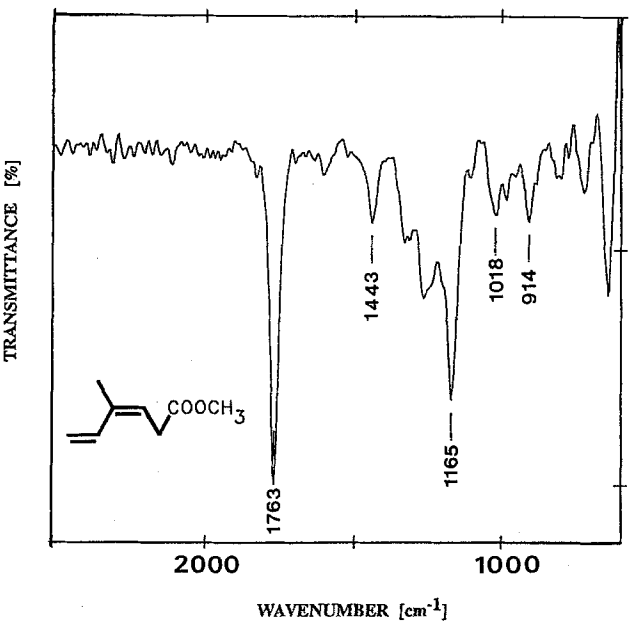
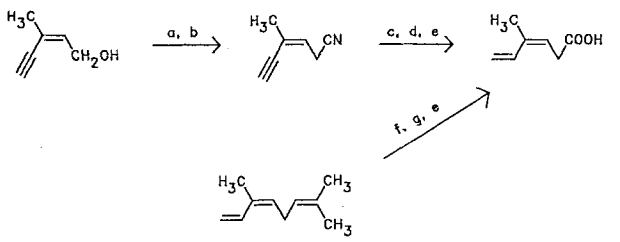


Figure 2. GC-FTIR spectrum of esterified authentic 4-methyl-3Z,5-hexadienoic acid.



a) imidazol/$P(C_6H_5)_3/J_2$, b) KC≡N/18-crown-6, c) MeOH/HCl (25%, w/w)
d) Zn(*Cu/Ag*)/MeOH, e) pig liver esterase/1N NaOH, pH=7, f) O_3/MeOH,TCNE, g) H_2SO_5/MeOH

Figure 3. Synthetic routes to the *Urophora* pheromone 4-methyl-3Z,5-hexadienoic acid.

vinyl group. Thus, in accordance with the MS- and IR-data, the natural product has to be a conjugated 4-methylhexadienoic acid with the two double bonds located at the aliphatic terminus. For this molecular framework only three arrangements are possible, namely 4-methylidene-5-hexenoic acid[3], 4-methyl-3E,5-hexadienoic acid[4] and a still unknown 4-methyl-3Z,5-hexadienoic acid. Because the GLC retention times of their methylesters were different (Kovàts-indices), the first two compounds could be readily excluded. The third remained to be synthesized. This was accomplished as outlined in figure 3. A direct approach utilizes the selective ozonolysis of Z-ocimene (ca 80% Z-isomer) and reduction of the ozonides with tetracyanoethylene, followed by immediate oxidation of the very sensitive aldehyde with Caro's acid (H_2SO_5) in methanol[3] to give the more stable methylester (ca 80% Z-isomer) in low overall yield (ca 5%). Subsequent hydrolysis with pig liver esterase at pH = 7 liberates the desired acid without rearrangement of the double bonds. A more stereoselective route was achieved starting from commercial cis-3-methyl-2-penten-4-yn-1-ol. After conversion into an iodide[5] and treatment with cyanide under phase transfer conditions[6] the resulting nitrile is readily transformed into the methylester with MeOH/HCl (25%). Reduction of the triple bond with activated zinc[7] yields the pure cis-ester, from which the free acid is obtained as described before. The new compound is characterized by the following data:

IR (CCl_4): 3600-2400 (br., OH), 2970 (m), 1712 (s, C=O), 1413, 1260, 1100, 1012, 912 cm^{-1}.

^{1}H-NMR (250 MHz, $CDCl_3$ as internal standard): 1.88 (s, 3H, CH_3); 3.28 (d, 2H, CH_2); 5.22 (d, 1H, CH=H_2); 5.34 (d, 1H, CH=CH_2); 5.50 (t, br., 1H, =CH-CH_2); 6.70 (dd, 1H, CH=CH_2).

MS (70 eV): 126 ($M^{+\cdot}$, 32), 111 (12), 96 (8), 84 (33), 81 (75), 79 (100), 67 (14), 65 (19), 53 (57), 45 (58).

Morphology. Dissection of male and female *U. cardui* and *U. stylata* showed an evident sexual dimorphism with respect to the size of the rectal ampulla (fig. 4) which serves as a reservoir for the volatile substance and hindgut contents. The ampulla is a part of the hindgut, and it can be closed by muscles which are located at the transition between hindgut and rectal ampulla. The volume of the rectal ampulla of male *U. cardui* can be up to ten times bigger than the ampulla of the female. The four rectal papillae projecting into the lumen of the ampulla are of similar size in both sexes regardless of the volume of the ampulla. These morphological differences between sexes indicate differences in the function of the rectal ampulla and its contents. Far more females of both species were dissected than were analyzed, but in no case could traces of the volatile be detected in female rectal ampullae. Both chemical and morphological data clearly point out that only the males are able to produce the volatile and to store it in the rectal ampulla.

Quantitative analysis of the volatile. Even by means of gas-chromatographic analysis we could not prove the presence of the volatile in female rectal ampullae. The determination of the amount of the volatile in rectal ampullae of male *U. cardui* indicated two different phases during 21 days after emergence (fig. 5). The first phase starts with the emergence of the male adult (we could not detect the volatile in the larvae). From day 0 up to day 9 there is a steady increase in the titre. The second phase is characterized by a constant level of 4-methyl-3Z,5-hexadienoic acid, which accounts for 2% of the rectal ampulla content. The mean amount of volatile in 145 *U. cardui* males analyzed was 65 nmol per male.

Different rearing conditions in the lab (flies kept fed or unfed, single, pairwise or in groups of 3 individuals) led to another interesting result. The number of flies kept together did not affect the titre at all. It increased only slightly, but not significantly, when the flies had access to an aqueous honey solution. There was no difference in the mean amount of volatile between males caught in the field and laboratory-cultured males.

The interpretation of the quantitative results in *U. stylata* is more difficult, since only 37 out of 66 laboratory-reared males accumulated any acid in their rectal ampullae. Because of the small sample size it can only be noted that although the absolute amount of acid was much lower than in *U. cardui*, the mean concentration of volatile in the rectal ampulla was just slightly diminished (1.6%). The mean amount of volatile in 37 males containing the acid was about 9 nmol. It may be of importance to note that all unfed *U. stylata* males (8 specimens) had no detectable volatile.

Bioassays. Except for the tests with potential predators, the bioassays were conducted to test the intraspecific sexual effects of the rectal ampulla contents of male *U. cardui.* Experiments with marked and unmarked host plants could not establish any influence on stay and oviposition behavior of *U. cardui* females. The results of choice tests with the vertical glass Y confirmed this tendency (fig. 6; the ordinate represents the amount of individuals (%) which were attracted (+) or repelled (−) after the application of rectal ampulla content onto one arm of the Y. Point 0 on the abscissa is the time of the

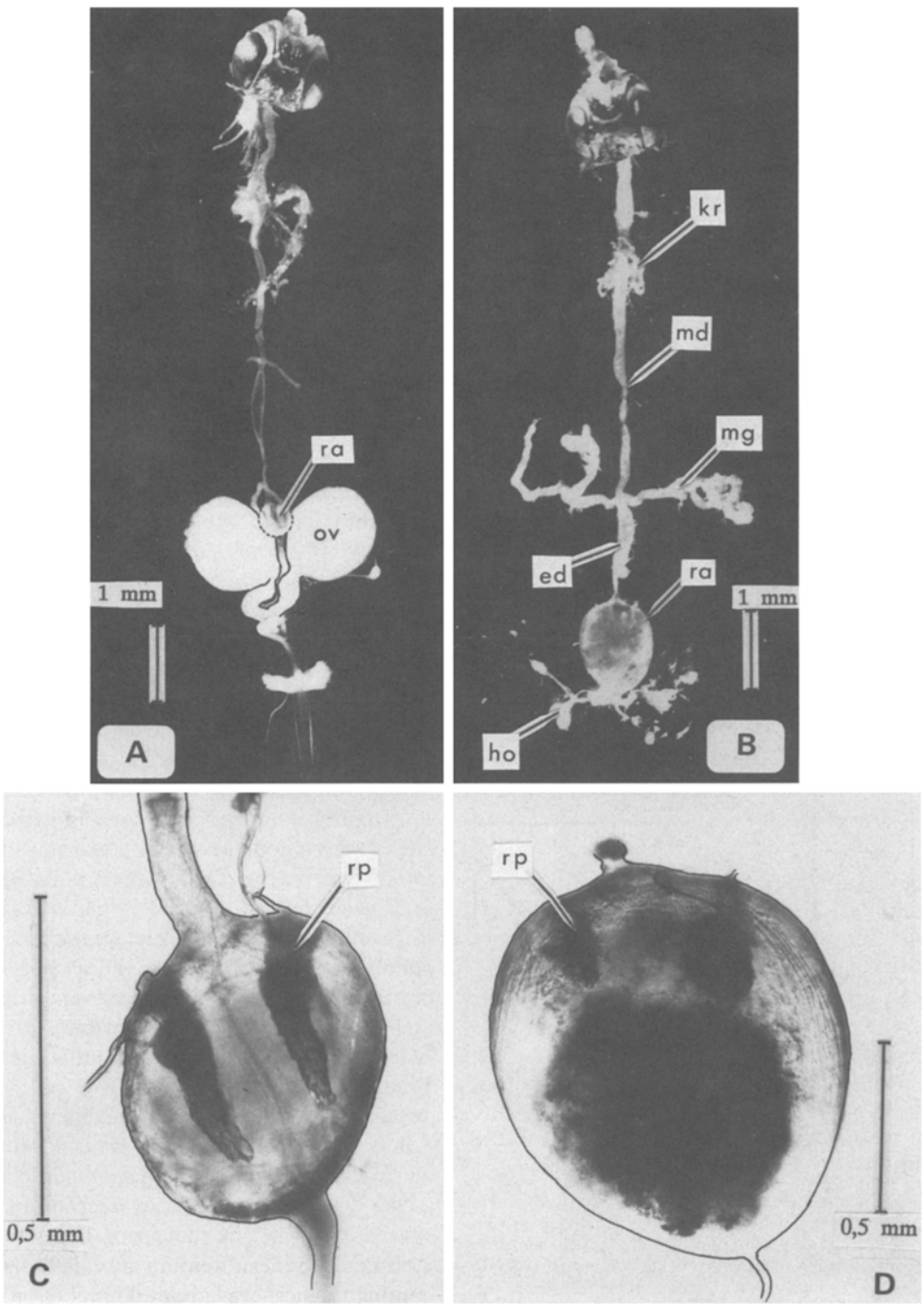


Figure 4. Morphology of the digestive apparatus in *U. cardui* females (A) and males (B); (C) rectal ampulla of the female; (D) rectal ampulla of the male; ed = hindgut, ho = testes, kr = base of the crop, md = midgut, mg = malpighian vessels, ov = ovary, ra = rectal ampulla, rp = rectal papilla.

control before the marking, point 1 the test immediately after the marking, 2.5 h and 3 h up to 4 h later. It is obvious that females were not attracted by male volatile in this experimental design. On the contrary, they seem to avoid the marking. Surprisingly, this tendency could be observed among the males too. At period 1 they are distinctly deterred by the marking (the confidence interval at period 1 does not include 0 on the abscissa). In the following periods the avoidance reaction decreases. This may be due to the chemical properties of 4-methyl-3Z,5-hexadienoic acid, because in the presence of oxygen a rapid autoxidation followed by polymerization takes place. The span of the confidence intervals indicates that although the mean reaction is always negative, an attraction during certain time periods is not unlikely. Our experiments concerning an interspecific role of the volatile

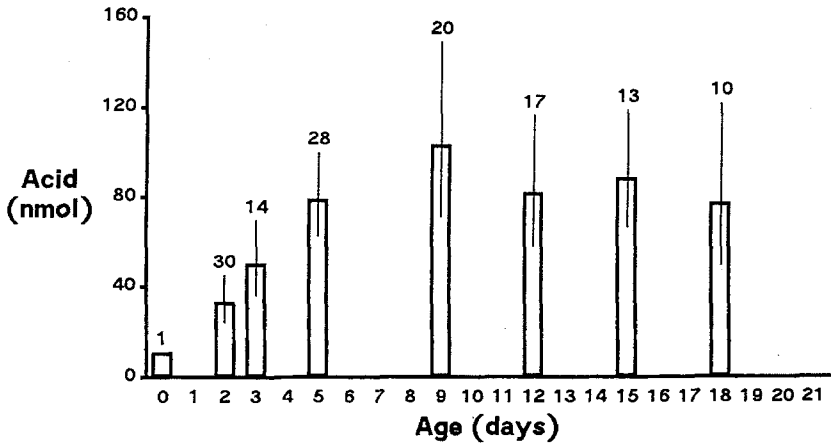


Figure 5. Quantity of 4-methyl-3Z,5-hexadienoic acid in rectal ampullae of 1 to 21-day-old males of *U. cardui*. Delogarithmed means (bars) and their corresponding confidence intervals (95%). The number of analyzed males for each data point is given above the confidence interval.

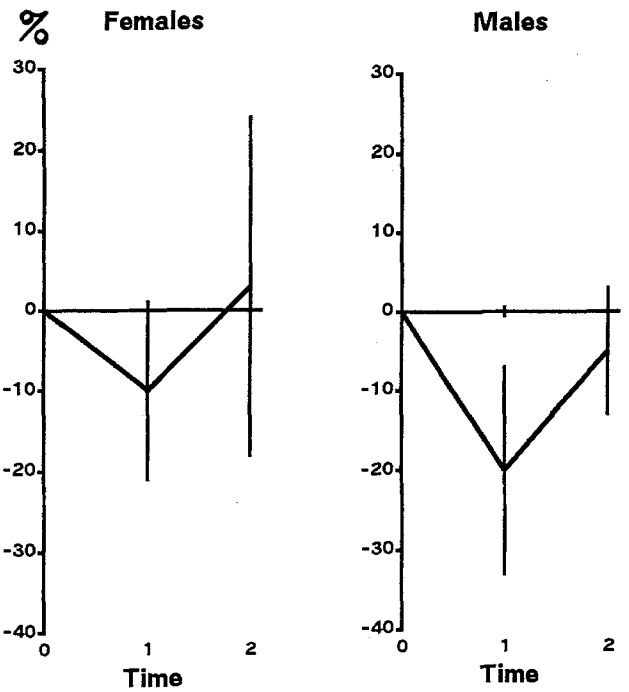


Figure 6. Y-test. The reaction of *U. cardui* males and females to rectal ampulla content of males is represented by the difference (%) in distribution in comparison to controls. Each point (0, 1, 2, 3) is the mean obtained from 3–15 tests. Vertical lines indicate confidence intervals (95%). (Females, period 3: lower limit – 58%; males, period 3: lower limit – 52%).

gave no proof of any repellent effect. In confrontation with syntopically occurring spiders and ants, neither males nor females of *U. cardui* seemed to be protected against their potential predators.

Discussion

Gland morphology. Three types of pheromone glands (**a**–**c**) are known in tephritids. Pleural glands (**a**) develop from enlarging epidermal cells only in abdominal segments 3,4,5 in male *Anastrepha suspensa* (Loew)[8, 9]. They were also found in *Toxotrypana curvicauda* Gerstaecker[10] and *Rioxa pornia* Walker[11]. Lhoste and Roche[12] described male specific internal anal glands (**b**) in *Ceratitis capitata* Wied. with spezialized sections of two anal papilla in the 7th abdominal segment[13], connected by ducts to the integument of the anal ampulla. During the calling behavior of the male the secretory products of the anal glands are dispersed by evagination of the anal ampulla.

Within the subfamily Dacinae, pheromone glands were investigated in several pest species. In all cases the typical site of pheromone production is a morphologically differentiated part of the rectal ampulla, the rectal gland (c_1). It consists of a secretory sac which develops as a finger-like evagination of the hindgut in the transition of rectal ampulla to anal tube and a reservoir that can be closed by muscles in order to prevent the mixing of feces and pheromone inside the rectal ampulla[14, 15]. The pheromone is passed through the anus during the calling behavior.

Although *Urophora* belongs, like *Ceratitis*, to the subfamily Trypetinae the site of volatile production and storage in male *U. cardui* and *U. stylata* is identical with that in the Dacinae. However, in *Urophora* a morphological specialization of the rectal ampulla into secretory and storing sections is absent. In the case of *Urophora*, we can consider that the whole male rectal ampulla acts as a rectal gland (c_2). This seems to be reasonable, because adults of *Urophora cardui* are most probably not dependent on food and contain at most traces of feces and excretions. Experiments in the lab showed that honey-fed flies had no significant advantage in terms of lifespan or the amount of volatile in the rectal ampulla. They seem to be nourished only by larval reserves stored in the fat body. It is obvious that the composition of feces of long-living species like the Dacinae (up to 460 days[16]) is different from that of the short-lived, probably non-feeding gall-forming *Urophora* (about 20–30 days). This assumption seems to be the reason why a selective release of volatile and feces is not realized in *Urophora*.

Quantitative analysis. The titre of 4-methyl-3Z,5-hexadienoic acid during three weeks of adult life in *U. cardui* males (fig. 5) resembles the pheromone titres in *Ceratitis capitata* males[13] and *Dacus oleae* females[17]. These studies showed that the maximum pheromone titre always corresponds with sexual maturity of the flies. Depending on age and number of mating periods, pheromone production has been found to increase rapidly after emergence and remain at a high level until senescence (male *Ceratitis capitata*[13]), otherwise the titre diminishes between two mating periods (female *Dacus oleae*[17]). A coincidence of maximum volatile content and sexual maturity does not apply to *Urophora*, since adults of both sexes emerge with developed reproductive organs and first copulations already occurred 12 h after emergence.

Bioassays. On the basis of the results of the bioassays we have to reject the arrestant hypothesis. A repellent function can be excluded too. Referring to a model of communication patterns in acalyptrate flies[18] the complexity of sexual signalling should be predictable by known facts about the ecology of a species. It is expected that the complexity of sexual signals increases when high encounter rates of sexual partners are not so certain. On the other hand signals can be very simple when encounter rates are high, as in the case of resource-based mating sites. This simplification tendency can reach the point of uncontrolled copulation attempts of males with any object of approximately the correct size and shape.

U. cardui and *U. stylata* belong to those species with resource-based encounter sites. They are monophagous gall-forming species and both sexes meet each other at definite rendez-vous places, their host plants, where copulation and oviposition takes place[1]. Several times we could observe that *U. cardui* males tried to copulate with other males. Even other species of *Urophora* were accepted as mating partners[19]. It is obvious that the ability for sexual discrimination is hardly developed within the genus. In the case of resource-based sites Burk[18] suggests a more important selection pressure for adaptations which enable a quick location of resources, a high defensive capacity against other males and a high probability for copulation with the females encountered.

According to this theory, and the biological data, signalling between sexual partners of *U. cardui* or *U. stylata* is unlikely. But if the volatile has a meaning at all, the Y-experiment might indicate that it acts in territorial defense between males. So the results of this experiment could lead to the following alternative hypothesis:

In order to defend a certain resource (host plant, female) against other males and to avoid expensive aggressive defense, males may have developed a simple pheromone system. The amount of dispensed pheromone might indicate the defensive capacity of a male. Since females seem to be deterred by the odor, too, a release of volatile should only happen in the absence of females or when it is more important to drive away a male than to attract a female. To establish a uniform or different reaction of males and females on the physiological level, exact measurements by electroantennogram methods could be useful. These methods should also be applied in further experiments which have to show whether this hypothesis about an intrasexual territorial function corresponds with reality.

Acknowledgments. The authors thank Prof. Dr H. Zwölfer and Dr R. Brandl (Animal Ecology I, University of Bayreuth, D-8580 Bayreuth, FRG) for many helpful discussions during this study and for reading the manuscript. We are also indebted to G. Schwinger (Basel) for high-resolution measurements of the volatile, Dr H. Diederich (Bruker analytische Meßtechnik GmbH, D-7500 Karlsruhe, FRG) for the GC/FTIR spectra.

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