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ORIENTAL FRUIT MOTH PHEROMONE: ATTRACTION OF FEMALES BY AN HERBAL ESSENCE

Ritsuo Nishida and Hiroshi Fukami

Pesticide Research Institute
College of Agriculture
Kyoto University
Kyoto, 606 Japan

Thomas C. Baker

Division of Toxicology and Physiology
Department of Entomology
University of California
Riverside, California 92521

Wendell L. Roelofs* and Terry E. Acree**

Department of Entomology*
Department of Food Science and Technology**
New York State Agricultural Experiment Station
Geneva, New York 14456

Introduction

The Oriental fruit moth, Grapholitha molesta (Busck) is one of the major pests of apples, peaches, plums, and other rosaceous fruits. The larvae damage the young twigs, causing them to die in early spring, and later generations feed inside the fruits rendering them undesirable. The female sex pheromone of the Oriental fruit moth has been established to be a mixture of Z- and E-dodecenyl acetates, Z-8-dodecenol and dodecanol (1,2). These are not unusual components for a female sex pheromone of a Lepidoptera. However, the entire sequence of courtship behavior in the Oriental fruit moth is unique among Lepidoptera in that males attract females after they themselves have been attracted to the vicinity of a

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female by her sex pheromone (3). This characteristic courtship sequence is shown in Fig. 1.

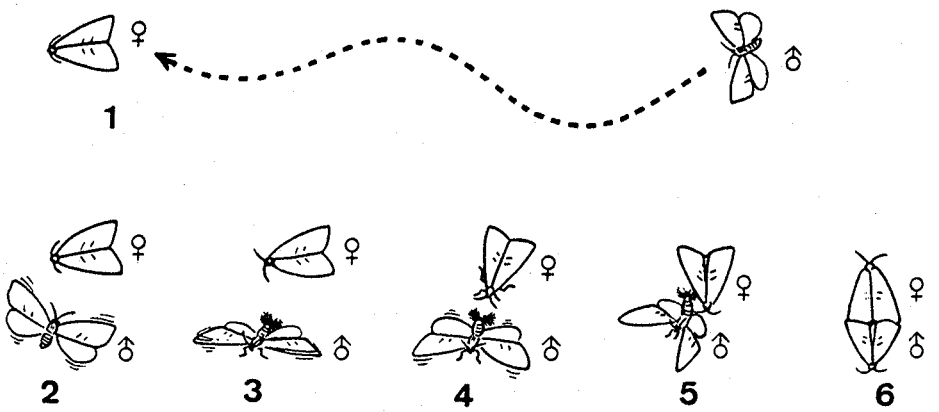

Figure 1. Courtship sequence of G. molesta.

Immediately after landing near the calling female [1-2], the male turns away and rhythmically extrudes and retracts his abdominal hairpencils, propelling volatile chemicals over the female using wind generated by his vibrating wings [3]. The female quickly walks toward the source of the odor and with her head touches the tip of the male's abdomen [4], evoking from him a copulatory attempt [5]. The male's wings rest on top of the female's wings [6]. The whole sequence of the courtship behavior provided a rare example of chemical communication between both sexes in insects.

The hairpencil organs are located between the 7th and 8th abdominal segments of the male moths, and are associated with the claspers. Each bundle of the hairpencils is composed of 93 hollow scales, and each scale is a honeycombed, porous matrix with channels. The scales are packed together in special pockets in the abdomen. When extruded, hairpencils emit a pleasant herbal or floral-like odor. The fragrant

chemicals of hairpencils have appeared to be responsible for the attraction of females. Here, we will review the isolation, identification, and behavioral and physiological functions of the hairpencil components of G. molesta (4,5).

Behavioral Assays

The emission of hairpencil volatiles is accompanied with wind puffs (90 cm/sec) created by wing vibration (3). Since this anemotactile stimulus also appeared to be an important factor in female attraction, the behavioral bioassay was conducted under a constant wind movement (71 cm/sec). Individual female moths (4 to 5 days old) were placed in a wind tunnel while calling on a sheet metal platform as shown in Fig. 2. One male equivalent of the extract or fraction, applied on a filter paper, was used as the test attractant. The behavioral response of females was scored "positive" in two grades if they walked upwind [1] and then touched the paper [2], and "negative" if they did not move at all during a 10-second exposure.

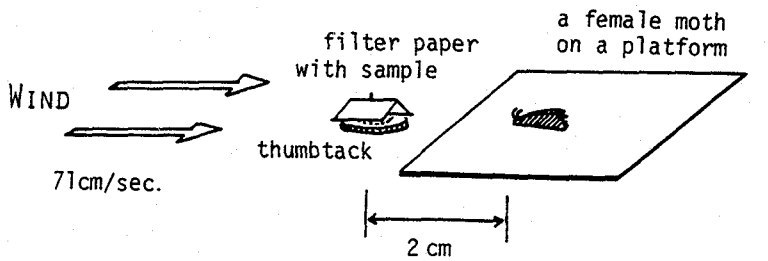

Figure 2. Behavioral bioassay of hairpencil pheromone extract to female G. molesta.

Isolation of Hairpencil Components

Approximately 5000 male equivalents (M.E.) of the hairpencils were used for isolation and identification of hairpencil components. Each paired hairpencils plus claspers from sexually matured males were snipped and immersed in a small vial of Skelleysolve B for several minutes, and the extract was then separated from the residue. This extract had retained the pleasant smell of the hairpencils. Fig. 3 shows the gas liquid chromatogram of the extract (3% OV-101 glass column, methyl silicone on 100-120 mesh Gas-Chrom Q, 2 m x 4 mm i.d., 150°C isothermal).

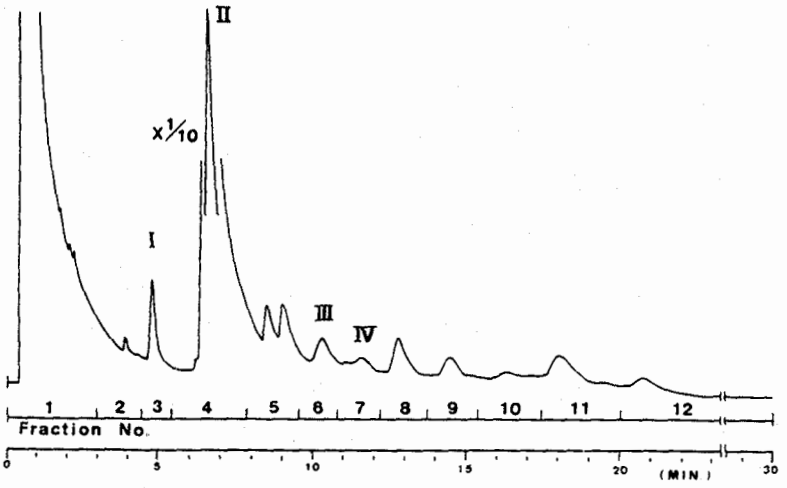

Figure 3. Gas chromatogram of male G. molesta hairpencil extract.

Among 12 fractions, only fraction 3 gave the significant behavioral response of upwind walking (29%; N=35). This fraction also gave the largest electroantennogram (EAG) response to the female's antenna as well (0.6 mV ± 0.2 SD; N=9). But the activity appeared to be much lower than that of the crude extract. However, a blend of compound I in fraction 3 with fraction 6 plus 7 produced as much female attraction

(80% upwind walking; N=40) as the crude extract. Fraction 6 plus 7 had the characteristic floral odor of the hairpencils. The herbal-scented compound IV in fraction 7 was found to be an isomer of Compound III in fraction 6. Despite its predominance in the chromatogram, compound II in fraction 4 was inactive by itself. These four components were isolated by preparative GLC under the same condition as given in Fig. 3. Compounds III and IV were further purified with XF-1150 (cyanoethyl methyl silicone on 100-120 mesh Chromosorb W-AW-BWCS, 2 m x 2 mm i.d.). The amounts of compounds I, II, III, and IV in a pair of hairpencil (1 M.E.) were estimated to be approximately 0.5, 20, 0.3 and 0.01 ng, respectively.

Identification of the Hairpencil Components

Compound I: This component, which showed both behavioral and electrophysiological activity, was identified to be ethyl trans-cinnamate from its diagnostic mass spectrum (M^+:m/z 176, 18%) and UV spectrum (lambda max 270 nm, epsilon 15000). Additional evidence was provided by the catalytic hydrogenation of I to ethyl 3-phenylpropanoate (M^+:m/z 178).

Compound II: The major component II was isolated in a crystalline form. The mass spectrum contained an apparent molecular ion at m/z 178 (64%) but was entirely different from that of dihydro-I. A UV spectrum of II exhibited 3 absorption bands (lambda max 210, 247 and 313 nm in ethanol) all of which showed a bathochromic shift in the presence of sodium hydroxide (lambda max 228, 252 and 350 nm, respectively), suggesting a 3,4-dihydroisocoumarin hydrogen-bonding with a phenolic proton (6). The NMR spectrum gave the conclusive evidence that compound II was mellein in which all 10 protons were resolved. The optical rotation of II gave a negative sign ($[\alpha]_D$ -133°, c=0.01 in $CHCl_3$), which confirmed that the absolute configuration was R (7).

Compounds III and IV: Both compounds exhibited essentially the same fragmentation (M^+:m/z 224), but only compound IV had the characteristic herbal odor. Compound III was identified as methyl jasmonate [methyl 3-oxo-2-(2-Z-pentenyl)-cyclopentyl-1-acetate] by comparison with an authentic sample (8). Compound IV was suggested to be a geometrical isomer or stereoisomer of compound III. The possibility that compound IV was the 2-epimer of methyl jasmonate was suggested by the fact that catalytic hydrogenation of both III and IV gave dihydro derivatives (M^+:m/z 226) with very similar mass spectra but different retention times and that IV could be readily converted into III in acidic media (9) and in a GLC injector above 160°C (III:IV = 95:5). The quantity of compound IV in the hairpencil, however, was too low for further analysis. Fortunately, the same herbal-scented compound was isolated in a larger quantity from lemon peels (10). Thus, compound IV was unequivocally identified to be methyl epijasmonate (11). Methyl epijasmonate is chiral, and only (+)-IV showed any sensory activity in humans (12). Therefore compound IV, which showed an extremely low odor threshold, should possess the 1S, 2R configuration (13). The structures of compounds I-IV are shown in Fig. 4.

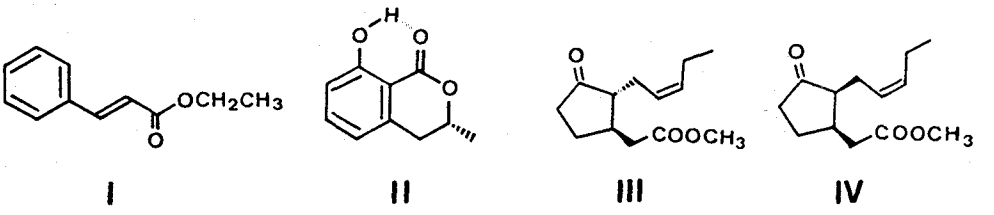

Figure 4. Structures of the four components from G. molesta hairpencils.

Biological Activity of the Hairpencil Components

Behavioral bioassays were carried out using 1 ng of each authentic sample in comparison with the crude hairpencil

extract (3 M.E.). The results are shown in Fig. 5. Ethyl trans-cinnamate (I) was the only compound to elicit a significant female attraction by itself, although it was less active than the crude extract in terms of the behavioral response. Since compound IV is inactive by itself and yet enhances the activity of I, it can be defined as a synergist. There was one alternative combination to elicit the female attraction: compounds I + II + III. We have not yet established whether it was due to a minor contaminant IV by epimerization of III.

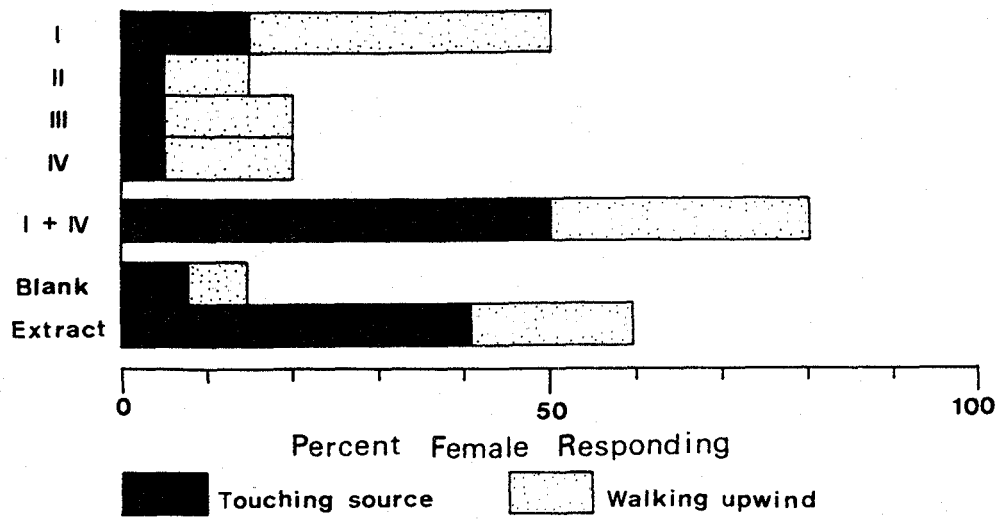

Figure 5. Behavioral response of G. molesta females to compounds isolated from male hairpencils. Treatments were 1 ng of each compound and 1 M.E. for the extract.

The EAG response of each hairpencil component was measured on both female and male antennae (Fig. 6). Ethyl trans-cinnamate was active on antennae of both sexes. Interestingly, a mixture of jasmonates, III and IV (93:7), did not give any EAG response. Mellein (II) elicited a significant response only in male antennae, which suggest a possible role of II in male-male communication, although the intensity was far lower than

that obtained from one of the female sex pheromone components, Z-8-dodecenyl acetate. The behavioral activity of mellein will be discussed in the next section.

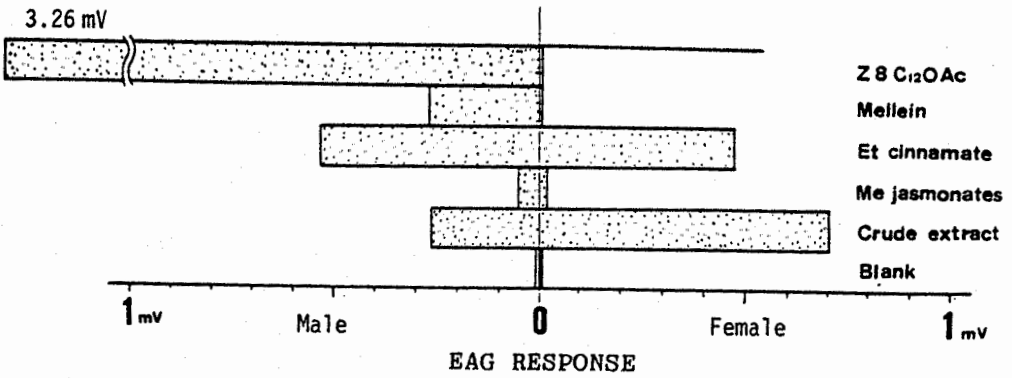

Figure 6. Mean EAG response amplitudes to the pheromone components.

Male Response to Mellein

The most abundant hairpencil volatile, R-(-)-mellein (II) showed no significant effect of female attraction, and also it was electrophysiologically inactive to the female antennnae. In contrast to these observations, the EAG assay showed a significant response to the male antennnae (Fig. 6). In order to examine the possible function of mellein in males, the authentic R-(-)-mellein was tested as a wind puff directed toward males that were resting on a screen of a male cage during the mating period. Each 2 ml of puff was directly blown (pointed) to the male head (about 5 mm away from the moth head) using the same glass pipets and syringe usually used for EAG assay. More than one-half of the males walked away from the source of the puff when the pipet was loaded with 100 ng of mellein, and at a higher dosage, males quickly walked away or flew away from the original position (Fig. 7). No such effect was observed when females were tested. Since a

pair of hairpencils contain mellein as high as 50 ng, this male reaction implies any role of mellein in male-male interaction. However, observation of G. molesta courtship indicated that males' displays frequently attract, rather than deter, other males (3). Further work would be needed to clarify the actual function of mellein along with other hairpencil components.

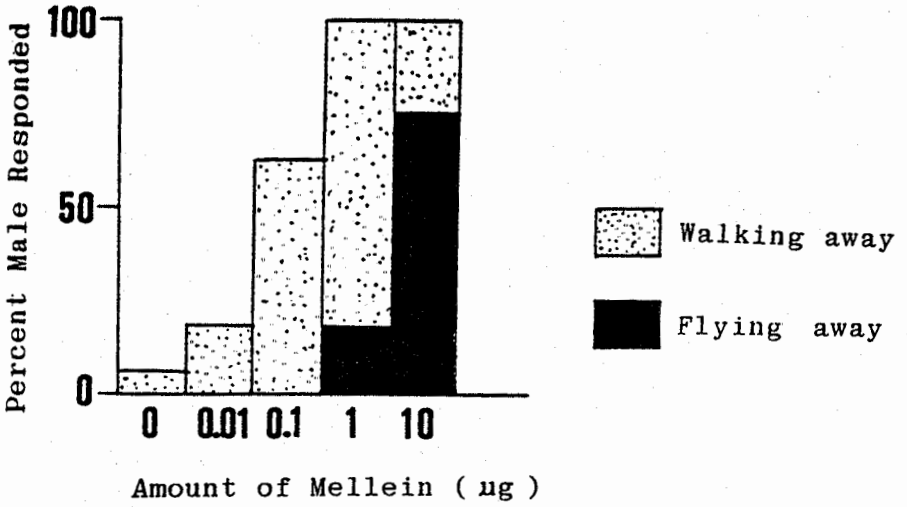

Figure 7. Behavioral effect of mellein to male moths.

Sequestration of the Hairpencil Components by Adult Males

Larvae of the Oriental fruit moth feed inside young fruits and twigs. Since both ethyl cinnamate and jasmonic esters and/or their derivatives are known plant constituents, it is possible that these chemicals are present in the host plant and are ingested and sequestered by the insect for later use. Preliminary studies indicated that hairpencils from the male Oriental fruit moths reared on an artificial diet do not possess the characteristic herbal odor of hairpencils. The hairpencil extract did not contain any detectable amount of I

on GLC analysis, whereas the major component, mellein (II) was present at a normal level. Varieties of the host fruits have been analyzed on GLC, but the contents of I, III and IV were below detectable levels. The biogenetic pathway of the hairpencil components during larval stage remains to be clarified.

On the other hand, we have found evidence that adult males have an ability to sequester a hairpencil component from their adult food. The adult moths (both sexes) sometimes visit injured fruits to ingest the juice. Bait traps with fruit juice or their fermentation products were used to attract the adult moths to monitoring traps before sex pheromone traps were introduced (14). Rotting fruits produce characteristic flavor components that differ from fresh fruits. Ethyl trans-cinnamate was found to be one of the components from rotting Japanese pears that actually attracted the Oriental fruit moths in the orchard. In order to examine the sequestration of the additional hairpencil components by adult males, we prepared an artificial diet. Since the moths will feed on sucrose solution, 15% sucrose solution containing 30 ppm of both pentadeutero-ethyl trans-cinnamate (d_5-I) and trideutero-methyl trideutero-epijasmonate (d_6-IV) (tri-substituted at the alpha-positions of the cyclopentanone) was offered to male moths. The large molecular ion peaks m/z 181 for d_5-I and 230 for d_6-IV along with the base peaks m/z 131 and 86, respectively, can be used as indices for the mass fragmentography of these compounds. Fig. 8 shows a mass fragmentgram of the hairpencil extract of the male Oriental fruit moths which were fed on the diet solution. The hairpencils were excised and extracted 4 hours after about 30 minutes of feeding. The major peak corresponds to mellein, and a fairly large peak containing m/z 181 and 131 fragments diagnostic to d_5-I. On the other hand, no significant amount of d_6-IV was detected although the diet contained the same quantity of d_6-IV as that of d_5-I. Thus, it seems that the male Oriental fruit moth is capable of absorbing from its diet, one hairpencil compound,

ethyl trans-cinnamate. Further work is now in progress using ^{14}C-labeled compound I.

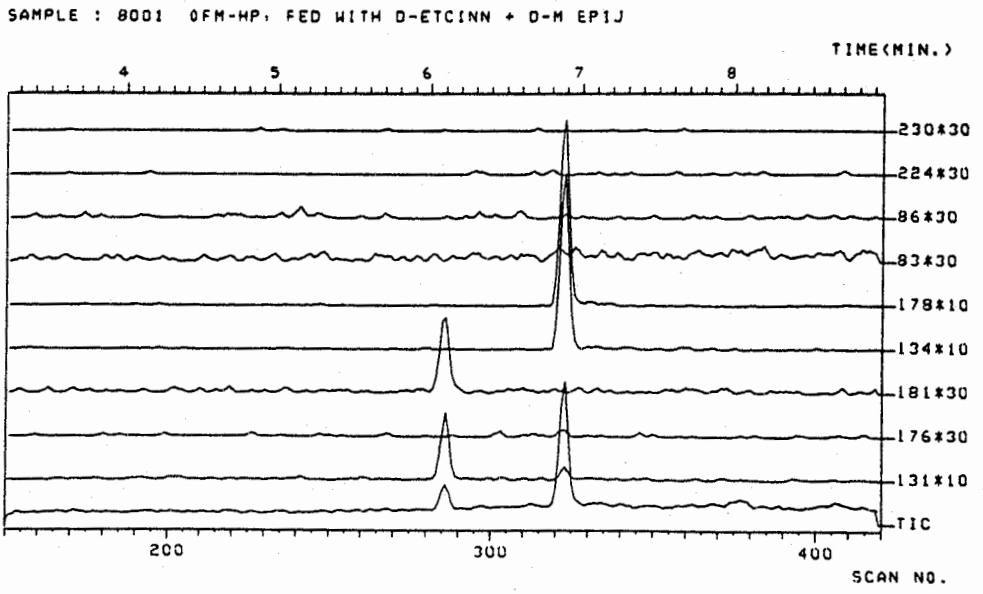

Figure 8. Mass fragmentgram of hairpencil extract after feeding on a diet solution containing d_5-I and d_6-IV. Retention time (min.) is given on an upper scale (I: 6.1, II: 6.9, III: 8.0, IV: 8.8).

Discussion

Courtship of the Oriental fruit moth involves a complex series of behavioral responses and corresponding signals sent in at least three sensory modalities, visual, anemotactile, and chemical, over a period of 1.5 seconds (3). The hairpencil display is released by a precise blend of female-emitted pheromone chemicals (2), and then female attraction is elicited by a male-emitted herbal essence. Here, a "chemical dialogue" between calling female and answering hairpencilling male has been fully established.

Males of many lepidopterous insects possess hairpencil-like organs, and evert and splay in the vicinity of females during courtship (15,16). However, the volatile chemicals from these scales usually exert a minimal observable effect on female behavior and the lack of an overt female response has hindered the identification of behaviorally active constituents. In rare instances in butterfly species, both behavior and chemistry have been elucidated, but the compounds described evoked female "acceptance" through inferred quiescence (queen butterfly, Danaus gilippus berenice, 17), or abdominal extension (sulfur butterflies, Colias eurytheme and C. philodice, 18) rather than attraction. Most other studies have identified the volatile chemicals extracted from hairpencils (19-30) or other specialized scales (31-33) without knowledge of the behavior these compounds elicited. Other studies attempted to define the behavioral roles played by scent through ablation (34-43), trapping experiments (44,45) or observation inference (46-48).

The four identified compounds from the Oriental fruit moth hairpencils have heretofore not been found in the Lepidoptera. Ethyl trans-cinnamate is similar to 2-phenylethanol, which has been found in the hairpencils of a number of lepidopteran species (23,28,29,49). Mellein is a known fungal metabolite (50-54) and has been found in a number of ant species (55). Methyl jasmonate is a constituent of jasmine oil and known in the perfume industry (8,56). It closely resembles cis-jasmone, which was identified from male Amauris ochlea butterfly hairpencils (30).

Electrophysiological techniques (57) were used in determining the GLC area of activity later found to be a behaviorally active compound I. Female EAGs have been used in previous studies to measure responses to potentially behaviorally active male producing compounds (15,45,58-62). The major hairpencil substance II was EAG active only in males. Initial

behavioral experiments implied that mellein might be a repellent in the male-male interaction during courtship. Similar observations of male-male interaction by hairpencil components have been reported in the case of Ithomiinae butterflies (32,63) and Pseudodaletia separata (64). Baker has recently described the variation in male Oriental fruit moth courtship patterns due to male competition, in which a late-arriving male sneaks copulations or disrupts the first male's courtship to succeed in mating (65). These observations have suggested that the late-arriving male can discriminate the first-arriving male near the female. Since the courtship sequence involves a complex series of behavioral responses, we have not yet elucidated whether the hairpencil chemicals actually trigger any competitive reaction between males.

Weatherston and Percy postulated the biosynthetic pathway of 2-phenylethanol from phenylalanine (16). One possible route involves the deamination of phenylalanine to give cinnamic acid, a common biosynthetic step in higher plants (66). It has not been demonstrated whether the Oriental fruit moth courtship pheromone components are synthesized in vivo from such primary substances, or whether intact pheromone components or closely related precursors are ingested and sequestered through to adulthood. Danaidone, known to increase mating success in Danaus gilippus males (17) and apparently also a constituent of the hairpencil secretion of D. chrysippus (21), is probably biosynthesized from dihydropyrrolidizine alkaloids, which D. chrysippus adult males ingest when visiting dried Heliotropium plants (67). Although male Oriental fruit moths are known to possess hairpencil pheromone components without ingesting fruit juice, we could demonstrate that the males have the capability to sequester additional ethyl trans-cinnamate from the diet during adulthood. Most of the moths were captured within 2 hours after the mating period by traps baited with fruit juice (68). Since the hairpencils lose a significant amount of their

odorous components after one display, additional sequestration from the juice might be advantageous for the next display.

Pyrrolidizine alkaloids in dried Heliotropium plants are known to act as phagostimulants to Ithomiinae butterflies, a group in which males sequester a Heliotropium metabolite in costal hairpencils (69). The food attractancy of ethyl trans-cinnamate has not been examined, but it was electrophysiologically active to male as well as female antennae. Rigorous work would be needed to clarify the biological significance of sequestration of hairpencil pheromone components.

References

1. Roelofs, W. L., Comeau, A., Selle, R.: Nature 224, 723 (1969).
2. Carde, A.M., Baker,T. C., Carde, R. T.: J. Chem. Ecol. 5, 423-427 (1979).
3. Baker, T. C., Carde, R. T.: Ann. Entomol. Soc. Am. 72, 173-188 (1979).
4. Baker, T. C., Nishida, R., Roelofs, W. L.: Science 214, 1359-1361 (1982).
5. Nishida, R., Baker, T. C., Roelofs, W. L.: J. Chem. Ecol. 8, 947-959 (1982).
6. Blair, J. Newbold, G.T.: J. Chem. Soc., 2871-2875 (1955).
7. Arakawa, H., Torimoto, N., Masui, Y.: Liebigs. Ann. Chem. 728, 152-157 (1969).
8. Demole, E., Lederer, E., Mercier, D.: Helv. Chim. Acta 45, 675-691 (1962).
9. Fukui, H., Koshimizu, K., Yamazaki, Y., Usuda, S.: Agric. Biol. Chem. 41, 189-194 (1977).
10. Nishida, R., Acree, T.E.: J. Agric. Food Chem. (in preparation).
11. Tanaka, H., Torii, S.: J. Org. Chem. 40, 462-465 (1975).
12. Acree, T.E., Nishida, R., Fukami, H.: J. Agric. Food Chem. (in preparation).

13. Nishida, R., Acree, T.E., Fukami, H.: Agric. Biol. Chem. (in preparation).

14. Tanaka, F., Yabuki, T.: Jap. J. Appl. Ent. Zool. 22, 162-168 (1978).

15. Birch, M.: Pheromones, Birch, M.C., ed., Elsevier, New York, 115-134 (1974).

16. Weatherston, J., Percy, J.E.: Advances in Invertebrate Reproduction I, Adiyodi, K.G., Adiyodi, R.G., eds., Peralam-Kenoth, Karivellur, Kerala, India, 295-307 (1977).

17. Pliske, T.E,, Eisner, T.: Science 164, 1170-1172 (1969).

18. Grula, J.W., McChesney, J.D., Taylor, O.R.: J. Chem. Ecol. 6, 241-256 (1980).

19. Meinwald, J., Meinwald, Y.C., Wheeler, J.W., Eisner, T., Brower, L.P.: Science 151, 583-585 (1966).

20. Meinwald, J., Meinwald, Y.C., Mazzocchi, P.H.: Science 164, 1174-1175 (1969).

21. Meinwald, J., Thompson, W.R., Eisner, T.: Tetrahedron Lett. 38, 3485-3488 (1971).

22. Meinwald, J., Boriack, C.J., Schneider, D., Boppre, M., Wood, W.F., Eisner, T.: Experientia 32, 721-722 (1974).

23. Alpin, R.T., Birch, M.C.: Experientia 26, 1193-1194 (1970).

24. Culvenor, C.C.J., Edgar, J.A.: Experientia 28, 627-628 (1972).

25. Edgar, J.A., Culvenor, C.C.J., Robinson, G.S.: J. Aust. Entomol. Soc. 12, 144-150 (1973).

26. Edgar, J.A., Cockrum, P.A., Carrodus, B.B.: Experientia 35, 861-862 (1979).

27. Grant, G.G., Brady, U.E., Brand, J.M.: Ann. Entomol. Soc. Am. 65, 1224-1227 (1972).

28. Jacobson, M., Adler, V.E., Kishaba, A.N., Priesner, E.: Experientia 32, 964-966 (1976).

29. Bestmann, H.J., Vostrowsky, O., Platz, H.: Experientia 33, 874-875 (1977).

30. Pitty, R.L., Boppre, M., Schneider, D., Meinwald, J.: Experientia 33, 1324-1326 (1977).

31. Lundgren, L., Bergstorm, G.: J. Chem. Ecol. 1, 399-412 (1975).

32. Edgar, J.A., Culvenor, C.J., Pliske, T.E.: J. Chem. Ecol. 2, 263-270 (1976).

33. Honda, K.: J. Chem. Ecol., 6, 867-873 (1980).

34. Myers, J., Brower, L.: J. Insect. Physiol. 15, 2117-2130 (1969).

35. Birch, M.: Anim. Behav. 18, 310-316 (1970).

36. Grant, G.G.: Experientia 30, 917 (1974).

37. Grant, G.G.: Ann. Entomol. Soc. Am. 69, 445-449 (1976).

38. Gothilf, S., Shorey, H.H.: Environ. Entomol. 5, 115-119 (1976).

39. Hirai, K.: Appl. Entomol. Zool. 12, 347-351 (1977).

40. Thibout, E.: C.R. Acad. Sci. Paris 287, 1141-1144 (1978).

41. Ono, T.: Appl. Entomol. Zool. 14, 432-437 (1979).

42. Rutowski, R.L.: J. Comp. Physiol. 115, 75-85 (1977).

43. Rutowski, R.L.: J. Chem. Ecol. 6, 13-26 (1980).

44. Dahm, K.H., Meyer, D., Finn, W.E., Reinhold, V., Roller, H.: Naturwissenschaften 58, 265-266 (1971).

45. Finn, W.E., Payne, T.L.: Southw. Entomol. 2, 62-65 (1977).

46. Clearwater, J.R.: J. Insect Physiol. 18, 781-789 (1972).

47. Pliske, T.E.: Ann. Entomol. Soc. Am. 68, 143-151 (1975).

48. Barrer, P.M., Hill, R.J.: Internat. J. Invertebr. Reprod. 2, 59-72 (1980).

49. Alpin, R.T., Birch, M.C.: Nature 217, 1167 (1968).

50. Nishikawa, E.: J. Agric. Chem. Soc. Jpn. 9, 772-774 (1933).

51. Yabuta, T., Sumiki, Y.: J. Agric. Chem. Soc. Jpn. 9, 1264-1275 (1933).

52. Patterson, E.L., Andres, W.W., Bohanos, N.: Experientia 22, 209-210 (1966).

53. Aldridge, D.C., Galt, S., Giles, D., Turner, W.B.: J. Chem. Soc. C 1971, 1623-1627 (1971).

54. Cole, R.J., Moore, J.H., Davis, N.D., Kirksey, J.W., Diener, U.C.: J. Agric. Food Chem. 19, 909-911 (1971).

55. Brand, J.M., Fales, H.M., Sokoloski, F.A., MacConnell, J.G., Blum, M.S., Duffield, R.M.: Life Sci. 13, 201-211 (1973).

56. Demole, E.P.: Fragrance Chemistry, Theimer, E.T., ed., Academic Press, New York, 349-396 (1982).

57. Roelofs, W.L.: Crop Protection Agents, McFarlane, N.R., ed., Academic Press, New York, 147 (1977).

58. Schneider, D., Seibt, U.: Science 164, 1173-1174 (1969).

59. Grant, G.G.: Nature 277, 1345-1346 (1970).

60. Birch, M.: Nature 233, 57-58 (1971).

61. Payne, T.L., Finn, W.E.: J. Insect Physiol. 23, 879-881 (1977).

62. Schneider, D., Boppre, M., Zweig, J. Horsley, S.B., Bell, T.W., Meinwald, J., Hansen, K., Diehl, E.W.: Science 215, 1264-1265 (1982).

63. Pliske, T.E.: Ann. Entomol. Soc. Am. 68, 935-942 (1975).

64. Hirai, K.: J. Chem. Ecol. 8, 1263-1270 (1982).

65. Baker, T.C.: Experientia 39, 112-114 (1983).

66. Zenk, M.H.: Proc. II Mtg. Fedn. Eur. Biochem. Soc., 45-60 (1966).

67. Edgar, J.A., Culvenor, C.C.: Experientia 31, 1-2 (1975).

68. Ueno, S., Hirose, K., Miura, K.: Bull. Nagano Pref. Agric. Exp. St. (Japan) 2, 69-75 (1960).

69. Pliske, T.E., Edgar, J.A., Culvenor, C.J.: J. Chem. Ecol. 2, 255-262 (1976).