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IDENTIFICATION AND SYNTHESIS OF FEMALE SEX PHEROMONE OF ORIENTAL BEETLE, *Anomala orientalis* (COLEOPTERA: SCARABAEIDAE)

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Abstract—Females of the Oriental beetle, *Anomala orientalis* (Waterhouse), release a sex pheromone composed of a 9:1 blend of (*Z*)- and (*E*)-7-tetradecen-2-one. The double-bond position of the pheromone was determined by DMDS derivatization and interpretation of the fragmentation patterns produced by monounsaturated ketones. In a sustained-flight tunnel, males responded by flying toward female beetles and attempting to copulate with them. Both effluvium and whole-body extracts of OB females were analyzed, and the activity was found only in the airborne extracts. Flight-tunnel bioassays also showed that a synthetic 90:10 *Z*/*E* blend on a rubber septum was attractive and that the responses of males to this blend were equivalent to *Z* isomer alone, but much better than to the single *E* isomer.

Key Words—*Anomala orientalis*, Oriental beetle, Coleoptera, Scarabaeidae, sex pheromone, (*Z*)- and (*E*)-7-tetradecen-2-one, DMDS derivatization, flight tunnel.

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INTRODUCTION

In the United States, the Oriental beetle (OB), *Anomala orientalis* (Waterhouse), is also called the Asiatic beetle (Westcott, 1964). This species is a close relative of the Japanese beetle and Asiatic garden beetles, which form a group of very destructive pests in lawns, nursery plants, and forest trees. *A. orientalis* is presumably a native of the Philippine Islands (Tashiro, 1987) and widely distributed in the Asiatic and Malaysian countries (Essig, 1951). It was imported into the United States before 1908 and nearly destroyed all of the sugarcane in Hawaii. *Scolia manilae* (Ashm.), a digger wasp, was introduced into Hawaii from Philippine Islands in 1916 as the entomophagous parasite to prey particularly upon grubs of *A. orientalis* (Clausen, 1940; Essig, 1931; Westcott, 1964). After the OB was first discovered on the mainland United States in New Haven, Connecticut, by M.P. Zappe and B.H. Walden in July 1920 (Essig, 1931), its distribution gradually increased in the northeastern United States. Although the adult beetles do some damage by chewing the blossoms of flowers, the larvae kill grasses, especially of lawns, by eating the roots close to the soil surface (Arnett, 1985; Metcalf et al., 1951). It was reported that OB not only attacked various turf grasses, lawns, and nursery stock, but also was found on ageratum, cyclamen, iris, hollyhock, phlox, rose, bean, beet, onion, rhubarb, and strawberry (Westcott, 1964).

Although the OB has become a serious pest in the northeastern United States and other countries and treated with insecticides (Fleming, 1948), its sex pheromone communication system has not been reported. Thus, a project was initiated to determine if this species uses a sex pheromone for mate location. In our preliminary studies, we were surprised to find that at an elevated temperature ($>25°C$) during photophase, adult OB males responded strongly in the flight tunnel to the sex pheromone released from females by flying toward and touching the source. As our studies progressed, Dr. Leal provided us information and a synthetic sample of the sex pheromone of the Oriental beetle, *Blitopertha orientalis*, in Japan (Leal, 1993).

METHODS AND MATERIALS

Insects. Second- and third-instar larvae of OB collected from the Norwich Community Golf Course in Norwich, Connecticut, in October 1992 were stored individually in ~30-ml plastic cups containing soil and a small amount of grass seed for food. They were stored at 10°C for about six months. When adults were required, larvae were moved to a 25°C emergence room under a 16L:8D photoperiod, where they were checked daily until adults emerged (ca. 4–6 weeks). Upon emergence, imagos were sexed and moved to a rearing room under common environmental conditions (16L:8D, 25°C, and 50% relative

humidity). They remained there in their soil-containing larval rearing cups prior to testing in the flight tunnel.

Pheromone Collections and Aeration Apparatus. A total of 17 4-day-old female beetles were introduced into an aeration apparatus, which consisted of three three-neck glass bottles (500 ml) filled with a wetted Kimwipes tissue and connected to three Super Q (200 mg each, Alltech Associates, Inc. Deerfield, Illinois) traps (15 cm × 0.6 cm OD). The air was filtered with a charcoal (Activated Carbon, 6–14 mesh, Fisher Scientific) trap (7 cm × 1 cm OD) before being pulled through the apparatus with a water aspirator or a vacuum pump. Flow rate was controlled at ~1 liter/min. Female beetles were aerated continuously for several days (16 hr in the photophase at 27°C and 8 hr in the scotophase at 18.5°C). The Super Q traps were changed and tissues were wetted with water every 24 hr. The airborne volatiles were obtained by percolating each Super Q trap with four portions of glass-distilled hexane (0.5 ml/each), and the resultant hexane solutions were then combined and concentrated under a stream of nitrogen to a volume of about 100 μl. The concentrated airborne hexane extracts were fractionated on the GC. Each fraction was then rinsed with a total of 200 μl of glass-distilled hexane for the bioassays.

Female Whole-Body Extracts. Female OB whole-body extracts were obtained by rinsing 12 virgin females (approximately 6-days-old) with three portions of 100 μl of glass-distilled hexane. The extracts were then combined and concentrated to a small volume with a nitrogen stream.

Instrumentation. Isolation and purification of the active components were performed on a Shimudzu GC-8A packed column gas chromatograph equipped with a thermal conductivity detector and a modified chilled collector. Dry Ice was used to cool the collection tube. The collection tube was a 75-μl micropipet (VWR Scientific Inc., Philadelphia, Pennsylvania), which was cleaned by heating to 550°C in a muffle oven for 12 hr. Helium was used as a carrier gas at a flow rate of 30 ml/min. The TCD current was set up at 100 mA and the injector and detector temperatures at 220°C. The fractions were collected each minute from either a packed nonpolar column (3% OV-101 on 80/100 mesh, Supelcoport, 2 m × 0.5 cm OD, Supelco, Inc., Bellefonte, Pennsylvania) or a polar column (5% Carbowax 20 M on 100/200 mesh, Supelcoport, 2 m × 0.5 OD, Supelco). The oven temperature was programmed from 150°C to 210°C at 5°C/min and then held for 20 min. A Hewlett Packard 3390A integrator was used to record the GC traces.

Analytical work was carried out on a Hewlett Packard 5880A gas chromatograph equipped with an flame ionization detector (FID) detector, and a nonpolar SE-30 or a polar Carbowax Econo-Cap capillary column (30 m × 0.25 mm ID, 0.25 mm film thickness, Alltech Associates) in the splitless mode. The oven temperature was programmed at 100°C for 2 min, then 5°C/min to 220°C and held for 20 min. Nitrogen was the carrier gas and the flow rate was

2 ml/min. GC–mass spectrometry (GC-MS) was carried out with a Hewlett Packard 5890 gas chromatograph coupled to a HP 5970B Mass Selective Detector using the same SE-30 capillary column and conditions as above, but with helium as the carrier gas.

Chemicals. The two compounds identified and assayed as the sex pheromones were (*Z*)- and (*E*)-7-tetradecen-2-one. They were synthesized individually by a method described later in the structure confirmation section. The isomeric purity of both isomers was >99.5%, based on results with the capillary GC (FID). The starting materials, (*Z*)- and (*E*)-6-tridecen-1-ol, were purchased from Dr. S. Voerman at the Institute for Pesticide Research, The Netherlands.

Laboratory Bioassays. GC fractions and synthetic pheromones were assayed for activity in a flight tunnel (Miller and Roelofs, 1978) and by the electroantennogram (EAG) technique (Roelofs, 1984). Temperature and relative humidity in the flight-tunnel room were maintained at ~27°C and ~50% , respectively. The air flow rate was at ~0.4 m/sec. Males were tested during the photophase and at a light intensity of ~800 lux. All of the samples, including the whole-body extracts, crude airborne extracts, GC fractions, synthetic pheromones, and controls (solvent only) were placed at the upwind end of the tunnel on a piece of filter paper or a rubber septum, which were pinned vertically on a piece of cork placed on a metal platform at a height of 15 cm.

Male beetles were transferred from the rearing room to the flight-tunnel room, placed individually in a screen release case, and allowed to acclimate for at least 2 hr without any disturbance prior to a test. Male beetles were introduced into the tunnel by placing the release cage at the downwind end of the tunnel facing upwind on a 15-cm-high platform. One male was released for each assay and observed for a period of 5 min while responses were recorded. After each assay, the male was removed from the tunnel and a new male was introduced at the start of subsequent tests. All tests were replicated at least four times using a new male for each replication.

The live-female source consisted of three to six females (at least 5-days-old) held in a small screen cage at the upwind end of the tunnel and used as the standard pheromone source. The flight-tunnel results were analyzed by the adjusted significance levels for proportions ($P < 0.05$) (Ryan, 1960). In all cases, bars with the same letters are not significantly different.

The EAG responses of male antennae were displayed on a Tektronix 2212 digital storage and analog oscilloscope, and printed out on a Tektronix HC 200 9-pin matrix Tekprinter.

RESULTS AND DISCUSSION

Pheromone Isolation and Identification. Female whole-body extracts did not elicit a behavioral response in the flight tunnel, but the airborne extracts were very attractive to male OB. Effluvium collected with the aeration apparatus

was first fractionated on the GC with a nonpolar OV-101 column and fractions were assayed in the flight tunnel. Fractions were taken each minute up to 30 min, but only the 5- to 6-min fraction showed significant activity. This retention time corresponded to two hydrocarbon standards—pentadecane (4.9 min) and hexadecane (6.5 min)—under these conditions. On the polar Carbowax 20 M column, an active fraction was found at 9–10 min retention time. The active fraction from the polar Carbowax column was further fractionated on the nonpolar SE 30 capillary column with fractions taken each minute for 30 min. Flight-tunnel and EAG bioassays showed that only a single GC peak at 15.67 min elicited a significant response. The amount of pheromone produced appeared to be the highest in 5-day-old females (Figure 1).

The active component was analyzed by GC-MS. The spectrum exhibited a relatively small molecular ion at *m/z* 210, with a base peak at *m/z* 43. In another GC-MS study, the selected ion monitoring (SIM) mode was employed to increase sensitivity. Three ions, *m/z* 125, 152, and 210, were selected from the mass spectrum as the monitoring ions characteristic of the natural pheromone, and they revealed two components at 15.49 and 15.61 min in a ratio of 88 : 12. This ratio varied to 96 : 4 with different collections. The results suggested that the natural pheromone consists of two geometric isomers. By comparison of the mass spectrum with unpublished data (at that time) on the Oriental beetle of the Japanese species, *Blitopertha orientalis* (Leal, 1993), the sex pheromone produced by female *Anomala orientalis* in the United States was also postulated to be (*Z*)- and (*E*)-7-tetradecen-2-one.

Confirmation of the double-bond location of the pheromone was obtained by capillary GC-MS analysis of DMDS (dimethyl disulfide) derivatives (Francis and Veland, 1981; Buser et al., 1983; Dunkelblum et al., 1985). Two sulfide fragments of the adduct appeared at *m/z* 159 and 145 (Figure 2a), indicating

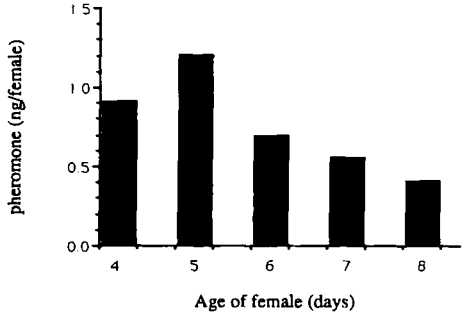


FIG. 1. Quantity of sex pheromone collected from 17 female Oriental beetles over a period of five consecutive days.

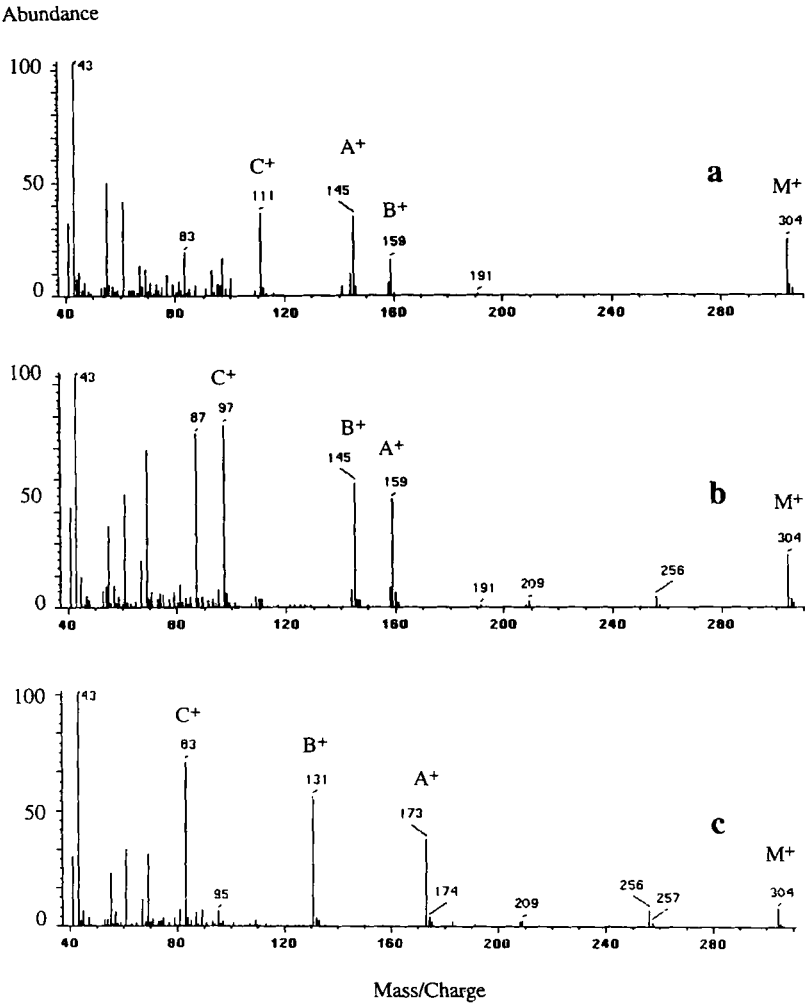


FIG. 2. Mass spectra of DMDS derivatives of three isomeric Δ-tetradecen-2-ones: (a) 7- (b) 6- and (c) 5-tetradecen-2-one. Molecular ion $M^+ = 304$. Fragment ions A^+, B^+, and C^+ are indicated.

that both 7- and 6-tetradecen-2-one were possible candidates for the natural pheromone.

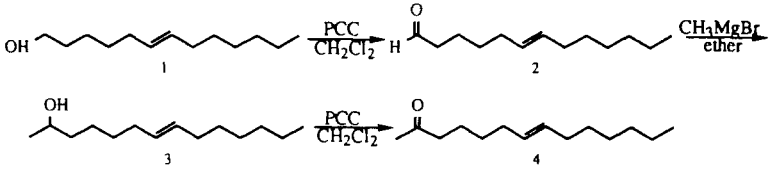
Chemical Synthesis and Structure Confirmation. To confirm the above formulation, the (*E*)- and (*Z*)-7-tetradecen-2-one isomers were synthesized indi-

vidually. The starting material, (*E*)-6-tridecen-1-ol **(1)**, was oxidized to the corresponding aldehyde **(2)** with 1.5 equivalent of PCC (pyridinium chlorochromate) in methylene chloride for 2 hr. The aldehyde was converted to (*E*)-7-tetradecen-2-ol **(3)** by treatment with 1.2 equivalent of methylmagnesium bromide in anhydrous ether. The final product, (*E*)-7-tetradecen-2-one **(4)** was obtained from **(3)** by oxidation with PCC. These reactions are summarized in Scheme 1. The *Z* geometric isomer, (*Z*)-7-tetradecen-2-one, was prepared by a similar pathway starting with (*Z*)-6-tridecen-1-ol.

The mass spectra of the synthetic pheromones and their DMDS adducts were virtually identical to those of the isolated natural products (*Z* and *E* isomers gave the spectra). The GC retention times on both the nonpolar SE-30 and polar Carbowax capillary columns also were identical with those of the natural pheromone. The larger and faster eluting peak observed with the pheromone on the nonpolar capillary column was assigned to the *Z* isomer.

Derivatization. The DMDS derivatives of biologically active GC fractions and synthetic samples were prepared according to standard procedures (Dunkelblum et al., 1985), with the exception of only a 4-hr reaction time. The observed fragments at *m/z* 159 and 145 in the mass spectrum of DMDS adducts of (*Z*)-7-tetradecen-2-one (Figure 2a) could also be produced from its isomer, 6-tetradecen-2-one, so it was necessary to conduct further investigations to make an unambiguous assignment of the double-bond location. For this purpose, (*Z*)-6-tetradecen-2-one and (*Z*)-5-tetradecen-2-one (gifts from Dr. Leal) were studied.

The diagnostic sulfide fragment ions A^+ and B^+ is the mass spectra of DMDS adducts of linear monounsaturated alkenes, esters, and acetates usually are the prominent peaks, and sometimes are the base peaks in their spectra. The relative intensities of ions produced by secondary fragmentation, such as fragments A^+-48 and B^+-48 (decomposed via loss of CH_3SH), are always smaller than their parent ions A^+ and B^+. The transaction of B^+-48, however, is found as the most predominant fragmentation in the DMDS adducts of these monounsaturated ketones compared to A^+-48. Therefore, the ions, B^+-48, can be used as the diagnostic fragments to locate the double-bond position in GC-MS



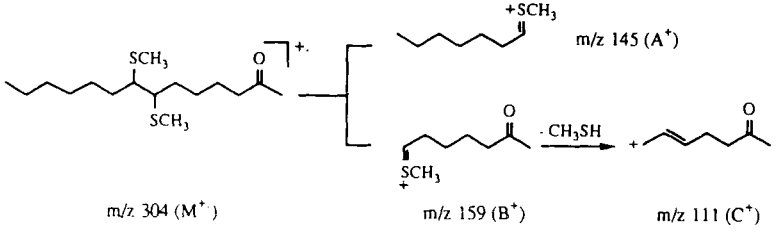
SCHEME 1. Synthetic pathway for Oriental beetle pheromone component, (*E*)-7-tetradecen-2-one.

analysis of DMDS derivatives of some monounsaturated ketones. The degradation of DMDS adduct of 7-tetradecen-2-one is illustrated in Scheme 2.

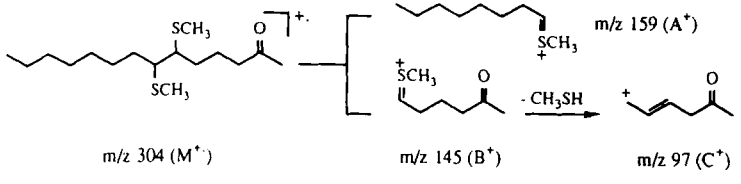
With this monounsaturated ketone, the relative intensity of the fragment ion, C^+ (*m/z* 111), is actually higher than its parent ion B^+ (*m/z* 159) (Figure 2a), whereas, fragmentation of $A^+ - 48$ (*m/z* 97) is not significant. Based on the fragment C^+ (*m/z* 111), the peak at *m/z* 159 can easily be assigned as the fragment B^+, and the double bond position in the natural pheromone can be unambiguously determined. The same degradation occurred in the mass spectra of DMDS derivatives of 6- and 5-tetradecen-2-one. The ion C^+ (*m/z* 97) is the second highest peak in the spectrum of the Δ6-isomer (Figure 2b). Even though the two sulfide fragments were similar to those obtained with natural pheromone, it is not difficult to determine that the ion at *m/z* 145 is the B^+ fragment (Scheme 3).

The $B^+ - 48$ fragment was also determined for the Δ5 isomer, which exhibits a capillary GC retention time that is identical with the natural pheromone. DMDS derivatization of (*Z*)-5-tetradecen-2-one produced a product with a molecular ion at *m/z* 304. Cleavage of the bond between the sulfur-substituted carbons led to two fragment ions, B^+ (*m/z* 131) and A^+ (*m/z* 173). The fragment C^+ (*m/z* 83) was obtained through further decomposition of B^+ with a loss of 48 units (CH_3SH) (Figure 2c). It was noted that in the case of the Δ5 and Δ6 isomers, the fragmentation of $A^+ - 48$ was not observed in the mass spectra of the corresponding DMDS derivatives.

The high intensity of the ion C^+, which is derived from the fragmentation



SCHEME 2. Degradation pathway of DMDS adducts of 7-tetradecen-2-one.



SCHEME 3. Degradation pathway of DMDS adducts of 6-tetradecen-2-one.

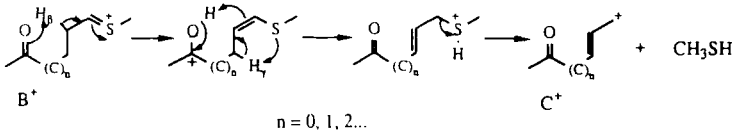
of $B^+ - 48$, suggests that the neutral molecule, methanethiol (CH_3SH), is much easier to lose from its parent ion, B^+, than from ion A^+. It is obvious that this fragmentation is formed via a hydrogen rearrangement and that the carbonyl group is involved in the rearrangement (Scheme 4).

The neighboring-group participation can be proposed to explain this phenomenon. Initially, hydrogen (H_β) could be transferred to the oxygen of the carbonyl group, and then$_\gamma$ extracted by a pair of lone electrons on sulfur to complete the 1,3 hydrogen migration. The key fragment, allylic ion C^+, would finally be formed by elimination of methanethiol (CH_3SH) as a neutral species from the resultant. The change transfer could be the driving force in this fragmentation. The number of CH_2 can vary at least from 0 to 2. This differs from the McLafferty rearrangement, in which the transferred hydrogen is restricted to the γ position to the carbonyl oxygen as it forms a six-member ring transition state.

Laboratory Bioassays. A series of behavioral tests were conducted in the flight tunnel to monitor the activity of GC fractions from airborne collections, to verify the activity of synthetic pheromones, and to evaluate the response of male beetles to different *Z*/*E* ratios. When live 5-day-old females were placed in the emission screen cage and used as the pheromone source, males exhibited a directed upwind anemotaxis toward the source, but the males did not show any response to a blank control in all assays performed.

The sequence of behavior exhibited by males to synthetic pheromone was comparable to the responses to caged females. The initial activation response of males was manifested by restlessness, waving of antennae with open lamellae directed upwind, waving of forelegs, walking upwind, and preliminary wing extension. The oriented upwind flights were started with taking off from the platform, performing a wide zigzag flight pattern, and locking on to the plume and proceeding in upwind flight. Finally, the males approached and landed on the emission screen cage (filter paper or septum when active GC fractions or synthetic pheromones were used as the sources) and attempted to copulate with the source. This behavioral response sequence is very similar to that described for another scarab, the green June bug (Domek et al., 1990).

The entire upwind flight sequence was not completed by some males, especially those less than 4 days old. Bioassays with males of different ages, using three to six live females as the source, showed that males younger than



SCHEME 4. Mechanism of elimination of methanethiol from fragment B^+.

4 days old did not fly, although some did activate. A batch of males was tested from their first day after emergence up to their ninth day using the different pheromone sources (three to six live females, 10 ng of *Z* isomer and 50 ng of *Z* isomer on filter paper). The results indicated that a significantly lower proportion of these males activated on their first to third day and that none flew to the source. With increasing age, greater numbers of males activated, took flight, and eventually touched the source (Figure 3). Therefore, only males that were more than four days old were used in the rest of flight tunnel tests.

Male OB responses were observed to various dosages and ratios of synthetic isomer. The isomers were located on the filter paper or rubber septa in different amounts and *Z*/*E* ratios and tested separately. With the filter paper, responses of males were not significantly different with either 50 ng *Z* isomer or 10 ng *Z* isomer compared with a live female (six) source (Figure 4). However, responses to the *E* isomer were significantly lower than to the *Z* isomer. Male responses to individual isomers and a blend (50 ng *Z*, 50 ng *E* and 50 ng 88 : 12 *Z*/*E* blend) relative to the live females (four to six) are compared in Figure 5, in which the proportions are averages of three trials. In these tests, there was not discrimination between the *Z* isomer alone and the blend, although there was a significant difference between responses to either the *Z* isomer or the blend and to the *E* isomer in all stages of male response. Interestingly, one male did fly all the way to 50 ng of the *E* isomer source in these studies.

A dosage study with rubber septa also was conducted to determine a usable range for field tests, as well as for future in-depth flight-tunnel tests. The rubber

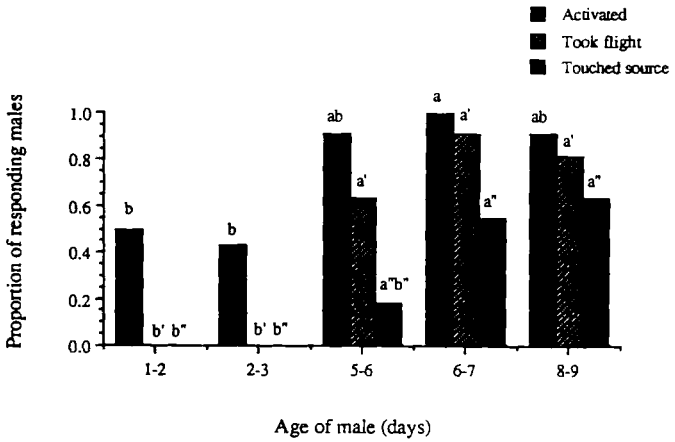


FIG. 3. Behavioral responses of OB males of different age to six live females. Bars (behaviors) superscripted by the same letter are not significantly different ($P < 0.05$. 1-2, $N = 10$; 2-3, $N = 14$; the rest, $N = 11$).

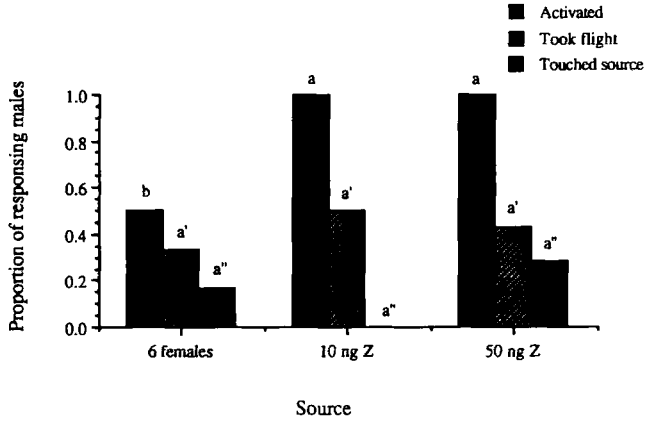


FIG. 4. Behavioral responses of OB males of different age to six live females, 10 ng *Z*, or 50 ng *Z* isomer. Separate comparisons were made for each behavior. Bars (behaviors) superscripted by the same letter are not significantly different ($P < 0.05$. Six females, $N = 6$; 10 ng *Z*, $N = 6$; 50 ng *Z*, $N = 7$).

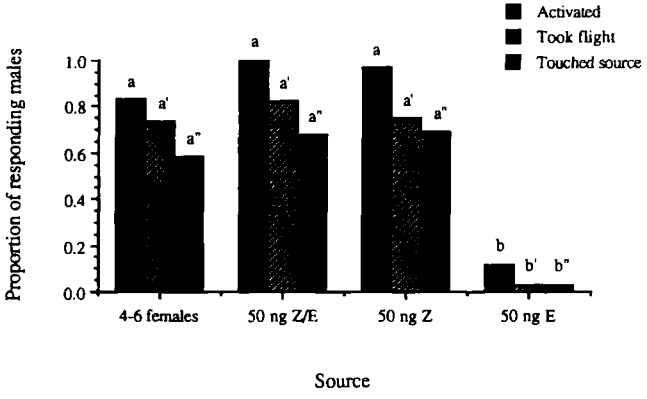


FIG. 5. Behavioral responses of OB males to a 50-ng source of the *Z*, *E* isomers and a 88:12 *Z*/*E* blend compared with four to six live females. Separate comparisons were made for each behavior. Bars (behaviors) superscripted by the same letter are not significantly different ($P < 0.05$. Trial 1, $N = 24$; trial 2, $N = 43$; trial 3, $N = 44$).

septa were baited with 1, 10, and 100 μg of a 90:10 *Z*/*E* blend. It was found that males were activated in significantly greater numbers to the 10-μg lure than to the 1-μg lure. Septa with the higher dosage (100 μg), however, elicited fewer completed flights. These data help to define the lower threshold level for com-

plete flight responses and the upper threshold level at which arrestment of upwind flight occurs in the flight tunnel.

The synthetic pheromone composed of a 90:10 *Z/E* blend (20 ng) elicited about a 1-mV EAG response with male antennae. The EAG value is comparable to the response of 17 female-day-equivalents of airborne collection.

Additional preliminary studies in the flight tunnel suggested that several other factors are important for OB behavior. First, virgin females in the emission cage were much more attractive than mated ones. Second, males older than four days flew to the pheromone source during photophase and scotophase. Third, when females were transferred from scotophase to the flight tunnel they did not elicit any male OB response during scotophase, although female beetles transferred from photophase did elicit complete responses from males during scotophase. These observations suggest that only virgin females OB release pheromone and they release it only during the photophase, or at least the calling period is initiated in photophase. These results are in agreement with the field-trapping studies with the OB (Facundo et al., 1994).

CONCLUSIONS

The sex pheromone isolated from female Oriental beetle, *Anomala orientalis*, was identified, using GC, GC-MS, along with flight-tunnel bioassays, to be a 96:4 to 88:12 mixture of (*Z*)- and (*E*)-7-tetradecen-2-one. The double-bond position of the pheromone (monounsaturated ketone) was determined by DMDS derivatization and interpretation of the diagnostic ions in their mass spectra. Behavior studies with male OB in the flight tunnel showed that 10 μg of a 90:10 *Z/E* blend on a rubber septum was an attractive source. The same pheromone with a similar *Z/E* ratio also was found in the Japanese Oriental beetle, *Blitopertha orientalis* (Leal, 1993). It is not known if these populations are the same species.

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