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Hesperophylax occidentalis (TRICHOPTERA: LIMNEPHILIDAE): ELECTROANTENNOGRAM STRUCTURE-ACTIVITY STUDY OF SEX PHEROMONE COMPONENT 6-METHYLNONAN-3-ONE

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Abstract—Electroantennogram (EAG) analyses of *Hesperophylax occidentalis* male and female antennae were used to generate dose-response curves for synthetic, racemic 6-methylnonan-3-one and to demonstrate the chemical specificity of male antennal olfactory cells. Male antennae responded to 6-methylnonan-3-one, the main female pheromone component. Females also demonstrated a response to 6-methylnonan-3-one, but a smaller one than males. The chemical specificity of male antennal receptors was determined by comparing EAG activity of synthetic, racemic 6-methylnonan-3-one and a series of structural analogs. The three structural parameters considered, including keto position, chain length, and methyl-group position, were demonstrated to contribute significantly to the reception of synthetic, racemic 6-methylnonan-3-one by male antennae. For straight-chain nonanones with keto groups at different positions, nonan-3-one elicited the greatest EAG responses from male antennae. For straight-chain alkan-3-ones with different chain lengths, the EAG responses of male antennae to nonan-3-one and decan-3-one were the same, but greater than those to other chain lengths. For methyl-branched nonan-3-ones, 6-methylnonan-3-one elicited greater EAG responses from antennae than analogs with the methyl group at another position.

Key Words—Caddisfly, limnephilid, aquatic insect, adults, EAG, attractant, antennae, analogs, chemical specificity, receptors.

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INTRODUCTION

Mayer and McLaughlin (1991) listed over 1300 insect species for which the sex pheromones or other attractants have been reported. These species comprise 10 orders, including the Trichoptera (Jewett, 1993; Löfstedt et al., 1994; Bjostad et al., 1995), Orthoptera, Homoptera, Hemiptera, Lepidoptera, Coleoptera, Mecoptera, Neuroptera, Hymenoptera, and Diptera (Mayer and McLaughlin, 1991). Despite its pervasiveness, the use of sex pheromones by members of the Trichoptera and other aquatic insects has received little attention until recently.

Wood and Resh (1984) first demonstrated chemically mediated sexual communication in the Trichoptera by trapping *Gumaga griseola* males with live, caged females and whole-body extracts. Chemical extracts of specific body sections from adult *G. nigricula*, *G. griseola*, and *Dicosmoecus gilvipes* females have also been used in field trapping (Resh and Wood, 1985). These experiments indicated that the fifth abdominal sternite of females contains the sex pheromone. Similar methods were employed to demonstrate sex pheromone use by the rhyacophilid, *Rhyacophila nubila* (Solem, 1985).

The identification of sex pheromones has been reported for several caddisfly species, including *Hesperophylax occidentalis* (Jewett, 1993; Bjostad et al., 1995), *Hydropsyche angustipennis* and *R. fasciata* (Löfstedt et al., 1994). *H. occidentalis* females produce a blend of compounds to which males demonstrate both an electrophysiological response in the laboratory and a behavioral response in the field (Jewett, 1993; Bjostad et al., 1995). Previous electroantennogram (EAG) experiments demonstrated that *H. occidentalis* males respond to extracts of the membrane between the fourth and fifth sternites in *H. occidentalis* females, but not to the sternites themselves. The pheromone blend was shown to include indole, 6-methylundecan-3-ol, 6-methylnonan-3-ol, 6-methylnonan-3-one, 6-methyloctan-3-ol, 6-methyloctan-3-one, 6-methylheptan-3-ol, and 6-methylheptan-3-one. Of these compounds, 6-methylnonan-3-one is present in greatest abundance and elicits the strongest EAG response from male antennae (Jewett, 1993; Bjostad et al., 1995).

The electroantennogram (EAG) has been a tool frequently used to study antennal receptor function for sex pheromones of moths and other insects. EAG response is assumed to be the summation of bioelectrical potentials generated by many olfactory receptor cells responding to odor stimulants (Nagai, 1984). The amplitude of the receptor potential of most insects is influenced not only by stimulus intensity, but by the chemical structure of the stimulus. The intersexual exchange of information by insects often transpires via molecules that differ only by their functional groups (Bestmann et al., 1987). Although receptor neurons are generally most active when exposed to the genuine pheromone, individual neurons may be excited by higher doses of a related analog. Male antennal neurons of many lepidopterans are generally receptive to pheromones

released by conspecific females or females belonging to closely related sympatric species (Priesner, 1979).

Interactions between pheromone molecules and their receptors have been investigated by analyzing the responses of identified receptor neurons to a homologous series of compounds (Boeckh, 1967; Kafka, 1970, 1974; Liljefors et al., 1985; Priesner et al., 1977; Schwarz et al., 1989; Vareschi, 1971; Dumpert, 1972; Kafka and Neuwirth, 1975). The literature is replete with structure–activity studies for lepidopteran, hymenopteran, orthopteran, isopteran, dipteran, and coleopteran pheromones (Prestwich, 1987). Analogs have been classified by chain length, functional group, regiochemicals with relocated double bonds, or carbonyls, diastereoisomers, and enantiomers (Prestwich, 1987).

The chemical specificity of *H. occidentalis* male antennae for 6-methylnonan-3-one was evaluated by comparing its EAG activity with that of several analogs. The analogs tested were different from 6-methylnonan-3-one either in keto position, chain length, or methyl-group position.

METHODS AND MATERIALS

Collection of Pupae and Preparation of Adults. Pupae were obtained during the summer and early autumn of 1990 and 1991 from a small stream in Hewlett Gulch, a tributary of the Poudre River (Larimer County, Colorado), or from the westernmost lake at Glacier Lakes approximately 5 km northwest of Centennial, Wyoming. Pupae in larval cases attached to the undersides of rocks by a silk thread were removed and placed into insulated coolers (4-liter capacity; Rubbermaid, Gott Corp., Winfield, Kansas) containing ambient water. Sufficient aeration was provided by battery-powered aquarium pumps (B&M Air Pump, West Point, Mississippi 39773) similar to those used by fishermen to maitain live bait. Pupae were returned to the laboratory where preparations were made for their eclosion.

Adult females frequently emerged several days later than males, but overlapping was observed. Behavioral studies suggest that caddisfly adults may be reproductively active following eclosion at either dusk or dawn (Williams and Feltmate, 1992). To prevent copulation and its consequent effects upon female production of or male response to pheromone that has been observed in some insects (McNeil, 1991), adults were removed from their styrofoam emergence containers immediately after eclosion and separated by sex.

The sexes of *H. occidentalis* adults were determined using their genital characteristics, abdominal color, and/or odor. Under a 25× low-power microscope, claspers that males use during copulation are visible. Parker and Wiggins (1986) present excellent illustrations of male and female genitalia. Abdomens of males were usually a pale green, while females could be identified by their

drab brown color. This characteristic is subject to geographical variation (Parker and Wiggins, 1985). Finally, the odor of compounds (to the human nose) secreted from a female's abdomen was very distinct, while the male displayed no detectable odor. Voucher specimens were deposited in the C.P. Gillette Museum of Arthropod Biodiveristy at Colorado State University, Fort Collins, Colorado 80523.

Synthetic Compounds. Octan-3-one, nonan-3-one, decan-3-one, and nonan-2-one were purchased from Aldrich Chemical Company, Inc. (1001 St. Paul Avenue, Milwaukee, Wisconsin 53233) (Figure 1). Nonan-4-one and 2-methylnonan-3-one were purchased from Wiley Organics (1245 6th St., Coshocton, Ohio 43812) (Figure 1). Dr. Louis Hegedus (Chemistry Department, Colorado State University) provided the nonan-5-one (Figure 1).

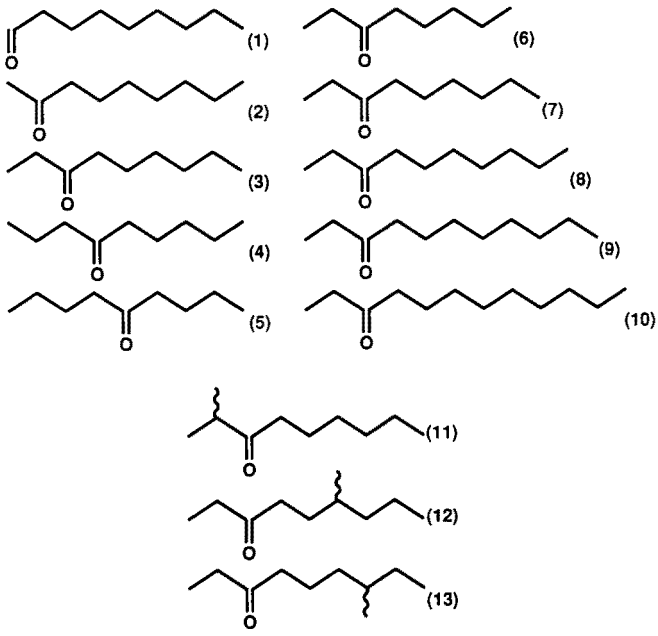

FIG. 1. Synthetic compounds used to characterize the specificity of male *Hesperophylax occidentalis* antennae. Squiggly lines represent a methyl group of undetermined stereochemistry; (1) nonanal, (2) nonan-2-one, (3) nonan-3-one, (4) nonan-4-one, (5) nonan-5-one, (6) octan-3-one, (7) nonan-3-one, (8) decan-3-one, (9) undecan-3-one, (10) dodecan-3-one, (11) 2-methylnonan-3-one, (12) 6-methylnonan-3-one, and (13) 7-methyl nonan-3-one.

The optical nature of the main sex pheromone component, 6-methylnonan-3-one, in *H. occidentalis* females is presently unknown. Many lepidopteran pheromones are optically active (Mayer and McLaughlin, 1991), which suggests a role for stereospecificity in the pheromone blend of *H. occidentalis* females. However, we did not have access to different optical isomers of 6-methylnonan-3-one, and only racemic mixtures were tested.

The racemic compounds 6-methylnonan-3-one, and 7-methylnonan-3-one (Figure 1) were synthesized from the racemic starting compounds 4-methylheptan-1-ol and 5-methylheptan-1-ol (Wiley Organics), respectively. Dodecan-3-one, and undecan-3-one were synthesized from decan-1-ol (Aldrich Chemical Company, Inc.), and nonan-1-ol (Aldrich Chemical Company, Inc.), respectively. Syntheses were completed by oxidizing 4-methylheptan-1-ol, 5-methylheptanol, decanol, and nonanol with pyridinium dichromate (PDC) (Corey and Schmidt, 1979) in $MeCl_2$ to form the corresponding aldehydes. A Grignard reaction with ethyl magnesium bromide (EtMgBr) in diethyl ether (Et_2O) was then performed to generate the secondary alcohols (Fieser and Fiesser, 1967). Reaction with EtMgBr was followed by another oxidation by PDC to generate the ketones. Final purification of the ketones was accomplished by their elution through a Florisil column with an equimolar mixture of $MeCl_2$ and Et_2O.

Synthesis of the racemic compounds 6-methylnonan-3-one, 7-methylnonan-3-one, dodecan-3-one, and undecan-3-one was modified from Corey and Schmidt (1979) and Fieser and Fieser (1967). The PDC reaction proceeded for 24 hr instead of 1 hr, with stirring provided by a 5-mm metal-impregnated glass rod. The remaining solid material was centrifuged to a small pellet at the bottom of the vial, and the final product in solution was transferred with a pipet to a clean vial.

Workup of the Grignard reaction was also modified. Excess EtMgBr was destroyed by adding to the reaction medium a water-saturated Et_2O solution. Destruction of excess EtMgBr was complete when water was no longer observed to react with it. The remaining white solid material was centrifuged to a pellet, and the Et_2O solution was pipetted to a clean vial, where it was extracted several times with distilled water. The final product was passed through a column of Florisil packed with Et_2O.

Treatment Preparations. Dose–response curves for males and females were obtained with five amounts of 6-methylnonan-3-one in $MeCl_2$: 0.1, 1.0, 10.0, 100, and 1000 μg, and a blank of 100 μl $MeCl_2$ that was evaporated to apparent dryness before testing.

In studies using synthetic analogs with different chain lengths, different keto positions, or different methyl-group positions, units of 200 μg were tested. All experiments included comparisons with 200 μg of synthetic, racemic 6-methylnonan-3-one as a positive control, and a blank of 100 μl $MeCl_2$ that

was evaporated to apparent dryness before testing. Preliminary experiments indicated that 200-μg doses elicited the maximum EAG response possible for each compound (for example, see Figure 2 below), which would tend to minimize differences in EAG responses due to differences in volatility among the compounds tested.

EAG Procedure. Because their life has not been observed to exceed five days under laboratory conditions, expedient use of the adult caddisflies was important. One antenna held at the base was plucked from the head with a pair of fine forceps. To increase exposed surface area and facilitate measurement of any depolarization, a 0.5-cm tip of the antenna was removed with a pair of thread trimmers and discarded. The remainder of the antenna was placed between two glass pipets filled with 0.15 M potassium chloride solution serving as electrodes. A few minutes were frequently required before testing to permit equilibration of the antenna and the KCl solution. Treatments were applied to separate 1×3-cm strips of filter paper from which the $MeCl_2$ was permitted to evaporate. The filter paper was folded lengthwise, slipped into a Pasteur pipet, and used immediately before active compounds could volatilize. Treatments were tested in random order. A 5.0-ml glass syringe was used to expel 0.5 ml air through the Pasteur pipet and across the antenna. A drop in potential across an antenna was enhanced by a $5000\times$ amplifier interfaced with a Commodore 64 computer (Commodore Business Machines, Wayne, Pennsylvania), and displayed graphically on a monitor (Bjostad and Roelofs, 1980; Bjostad, 1988). Fatigue was minimal throughout the duration of testing each antenna, as indicated by the small variation in responses to compounds tested.

Statistical Analyses. Data obtained from the electroantennograms were analyzed with SAS (SAS Institute Inc., SAS Circle, Box 8000, Cary, North Carolina 27512-8000). Experiments in which ANOVA yielded significant P values (0.05) were subjected to a comparison of total EAG activity with Newman-Keuls multiple-range test.

RESULTS

Both male and female antennae produced significant EAG responses to synthetic, racemic 6-methylnonan-3-one (Figure 2). The EAG responses of males to synthetic, racemic 6-methylnonan-3-one were greater than the EAG responses of females. Electrophysiological responses of both male and female antennae were directly proportional to the amount of synthetic, racemic 6-methylnonan-3-one applied to them (Figure 2). The amount of 6-methylnonan-3-one required to elicit a response from male antennae was four orders of magnitude less than that required for females; 1000 μg of 6-methylnonan-3-one was required to elicit a response from female antennae significantly greater than that elicited by a

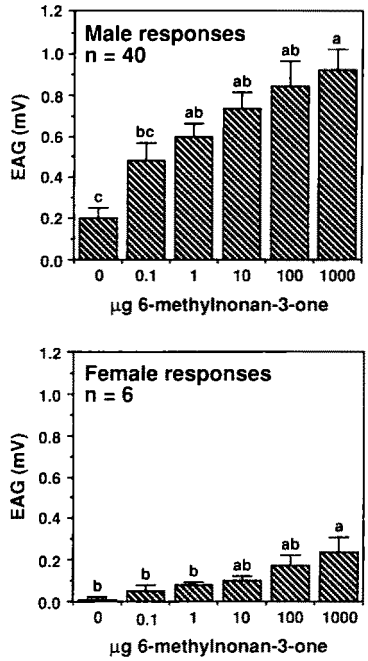

FIG. 2. Synthetic, racemic 6-methylnonan-3-one dose–response curves for *Hesperophylax occidentalis* males and females; n = number of responses to each treatment recorded from three male and three female antennae. Bars on columns represent standard errors. Treatments denoted by the same letter are not significantly different by Newman-Keuls multiple-range test ($P = 0.05$).

control of evaporated $MeCl_2$. The lowest dose of 6-methylnonan-3-one tested was 0.1 µg, and it elicited a significantly greater response from male antennae than the control.

Results of the EAG experiments were consistent with our prediction of chemical specificity for reception of 6-methylnonan-3-one by male antennal olfactory cells and support the importance of all structural parameters used. The responses elicited from male antennae waned with analogs whose structure deviated increasingly from that of the main pheromone component, 6-methylnonan-3-one. When considering the effect of different keto positions, there were also significant differences in the EAG activity elicited by analogs of 6-methylnonan-3-one (Figure 3). Multiple-range tests indicated that a significant difference exists between nonan-3-one and all other treatments, but no statistically significant differences exist among nonan-2-one, nonan-4-one, and nonan-5-one.

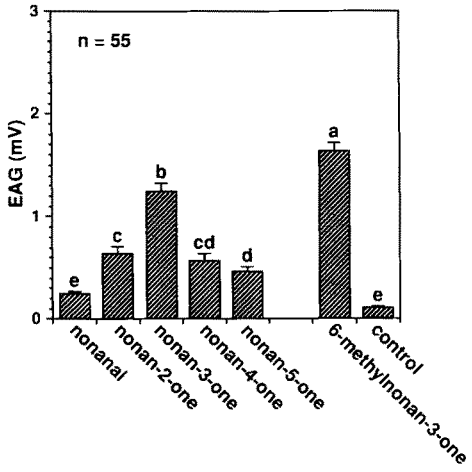

FIG. 3. *Hesperophylax occidentalis* male EAG responses to nonanones with varying keto positions (200 μg of each); n = number of responses to each treatment recorded from 11 male antennae. Bars on columns represent standard errors. Treatments denoted by the same letter are not significantly different by Newman-Keuls multiple-range test (P = 0.05).

Significant differences were obtained between nonan-5-one and nonanal and between nonanal and the control of evaporated $MeCl_2$. Responses elicited by 6-methylnonan-3-one were significantly different from all other treatments.

When considering the effect of different chain lengths, a significant difference among EAG activity elicited by analogs of 6-methylnonan-3-one was observed (Figure 4). EAG responses of male antennae to nonan-3-one and decan-3-one were equally strong. These two compounds elicited significantly higher responses than octan-3-one, which elicited significantly higher responses than undecan-3-one. Undecan-3-one elicited significantly higher responses than dodecan-3-one. No significant difference was observed between EAG responses to dodecan-3-one and the control of evaporated $MeCl_2$. The average response elicited from male antennae by 6-methylnonan-3-one in that experiment was significantly greater than all other treatments.

Significant differences were observed among the EAG activity of 6-methylnonan-3-one analogs differing in methyl-group position (Figure 5). Responses by male antennae to methyl-group position analogs were smaller than those of synthetic 6-methylnonan-3-one. Furthermore, the magnitude of the EAG responses was inversely related to the distance of the methyl group from the sixth carbon. 7-Methylnonan-3-one elicited a significantly larger response than

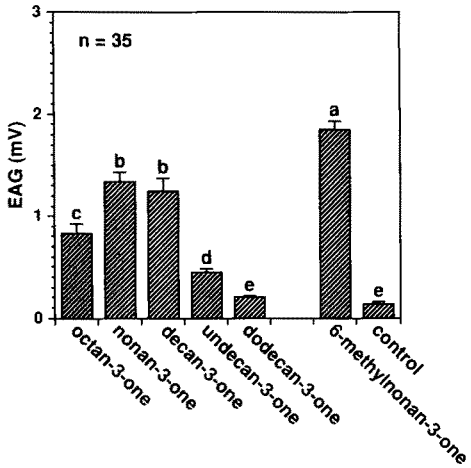

FIG. 4. *Hesperophylax occidentalis* male EAG responses to alkan-3-ones of varying length (200 μg of each); n = number of responses to each treatment recorded from 11 male antennae. Bars on columns represent standard errors. Treatments denoted by the same letter are not significantly different by Newman-Keuls multiple-range test (P = 0.05).

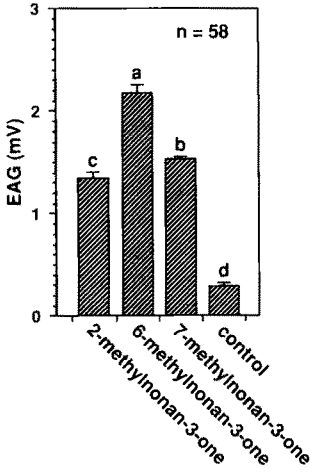

FIG. 5. *Hesperophylax occidentalis* male EAG responses to varying methyl-substituted nonan-3-ones (200 μg of each); n = number of responses to each treatment recorded from 12 male antennae. Bars on columns represent standard errors. Treatments denoted by the same letter are not significantly different by Newman-Keuls multiple-range test (P = 0.05).

2-methylnonan-3-one. Other commercially available methyl-branched alkanones were also tested as indicators of antennal specificity for the main pheromone component. 7-Methylnonan-3-one and 2-methylnonan-3-one elicited significantly larger EAG responses than 5-methyloctan-3-one. 5-Methyloctan-3-one elicited a response significantly higher than that elicited from 4-methylheptan-3-one. The activity elicited by 4-methylheptan-3-one was not significantly different from the control. The average male EAG response elicited by synthetic 6-methylnonan-3-one was significantly greater than that from all other treatments.

DISCUSSION

Results of our EAG experiments were consistent with previous observations that females produce compounds to which males have a highly selective response. Small structural changes in the molecule 6-methylnonan-3-one caused appreciable reductions in the receptor potentials of *H. occidentalis* male antennae. These reductions in receptor potential demonstrate that the olfactory cells of male antennae are selective for 6-methylnonan-3-one. The structural parameters demonstrated to be responsible for this selectivity are keto position, chain length, and methyl-group position. The consequences of selectivity by olfactory cells are illustrated by our experiment comparing the electrophysiological activity of chain length analogs. EAG responses of male antennae to nonan-3-one and to decan-3-one were the same, consistent with our expectation that the putative receptor for 6-methylnonan-3-one is specialized for a compact molecule that is sterically intermediate between nonan-3-one and decan-3-one.

Previous work has demonstrated that similar structural alterations in pheromone components significantly reduce EAG activity, indicating that receptors on insect antennae characteristically have limited flexibility. For sex pheromones of moths, it has been demonstrated that chain length, double-bond position, double-bond configuration, and terminal functional groups (typically alcohol, aldehyde, or acetate) all influence the magnitude of EAG responses (Roelofs, 1984). Structure–activity studies with analogs of (*E*,*Z*)-2,13-octadecadien-1-yl acetate, a main pheromone component of the currant clearwing moth, *Synanthedon tipuliformis*, indicate that hydrophobic, steric, and electronic effects of chain terminal groups are important physical parameters responsible for biological activity of the compound (Hoskovec et al., 1993).

Chain length of sex pheromone analogs was an important determinant of EAG activity in our work with *H. occidentalis* and has been considered elsewhere. Electrophysiological recordings from single receptor cells were obtained from antennae of *Agrotis segetum* in response to chain-length analogs of the main sex pheromone component (*Z*)-5-decan-1-yl acetate, and strong reductions

in elicited potentials were observed with chain lengths other than 10 carbon atoms (Liljefors et al., 1985). Single-sensillum studies indicated that the terminal methyl group and the acetate group of (*Z*)-5-decenyl acetate interact with highly complementary sites in the receptor cavity (Bengtsson et al., 1990). Furthermore, the magnitude of responses decreased with the elimination of methylene units.

With respect to alkyl substituents, Jönsson et al. (1991a, 1993) found that methyl groups placed at different locations in (*Z*)-5-decenyl acetate resulted in a significant reduction of EAG response. Methyl-substituted analogs of the pheromone for the turnip moth were synthesized and used to study the degree of steric complementarity between the acetate-substituted alkyl chain of the pheromone component and its receptor (Jönsson et al., 1991a,b). Fescemeyer and Hanson (1990) introduced either a cyclopropyl or *tert*-butyl group at the terminal portion of (*Z*)-11-tetradecen-1-ol acetate, the main pheromone component of the European corn borer moth *Ostrinia nubilalis*, and they found that EAG responses of males were either significantly higher or lower than responses to the main pheromone component itself, depending on the genetic strain tested.

We have not yet determined the enantiomeric blend of 6-methylnonan-3-one that is involved in pheromone communication in *H. occidentalis*. The EAG technique can play an important role in determining relative antennal response activity among optical isomers that cannot be characterized with minute quantities of natural material (Roelofs, 1984). EAG studies demonstrated that the highest responses occurred selectively with the correct enantiomer of 4-methylheptan-3-ol, the trail pheromone of the ant *Leptogenys diminuta* (Kern and Bestmann, 1993). Studies with the American cockroach *Periplaneta americana* indicated that EAG responses were higher with the correct enantiomer of periplanone-A than with an optically active analog (Okada et al., 1990). *R* and *S* enantiomers of methyl-substituted compounds elicited different EAG responses from male *Agrotis segetum* for some methyl-group positions, but not all (Jönsson et al., 1993). EAGs were used to provide insight into the enantiomeric blend of the aggregation pheromone used by both *Pissodes strobi* and *P. nemorensis* (Hibbard and Webster, 1993). In that study, responses of *P. nemorensis* antennae to (1*R*,2*S*)-grandisal were significantly higher than to (1*S*,2*R*)-grandisal. EAG analyses demonstrated that the 9*S*,10*R* enantiomer of (*Z*,*Z*)-3,6-*cis*-9,10-epoxyheneicosadiene elicits a male antennal response two to three times greater than the 9*R*,10*S* enantiomer from *Hyphantria cunea* and *Estigmene acrea* (Hill et al., 1982).

Kentaro et al., (1991) studied the EAG responses of cockroaches to periplanone and four groups of analogs. The analogs were those containing a germacrene skeleton, those without the *exo*-methylene and isopropyl groups on the 10-membered ring, compounds similar to periplanone, but with altered chain length, and the four candidates of periplanone-A (P-A) including Hauptmann's

P-A, Macdonald's P-A, Persoon's P-A, and its epimer. Hauptmann's P-A elicited the strongest responses of the four analogs, suggesting that it is a natural P-A produced by female cockroaches.

EAGs provide useful information on the specificity of insect olfactory receptors, but recordings from individual olfactory cells are necessary for a detailed understanding of the perception of odor molecules by insects (Wadhams, 1984). For example, in a comparison of EAG responses and single sensillum responses of the sugar beet moth (*Scrobipalpa ocellatella*), Renou et al. (1980) found that the responses to a series of pheromone analogs were almost identical, but the single sensillum recordings showed that responses from two cell types contributed to the EAG profile. Similar specificity of single cells for one compound (or a small set) has been found in other species as well. Single-unit recordings from sex pheromone antennal receptors in the moth *Antheraea polyphemus* indicated that one antennal receptor type responds to the acetate pheromone component, but not to the aldehyde component, and a second antennal receptor responds selectively to the aldehyde component (Bestmann et al., 1987). Single-sensillum recordings from *Yponomeuta rorellus* males revealed two types of cells in most sensilla (Löfstedt et al., 1990). A large spike amplitude cell was stimulated by tetradecyl acetate and the unsaturated analogs (*E*)-11-, (*E*)-6-, and (*E*)-12-tetradecenyl acetate, and to a lesser extent by the (*Z*)-11, (*Z*)-6, and (*Z*)-12 isomers. A cell with medium spike amplitude was stimulated by (*Z*)-9-tetradecenyl, and (*Z*)-11-hexadecenyl acetate. Some sensilla contained a third cell firing with a small spike amplitude, which was activated by (*Z*)-11-tetradecenol.

The dose–response curve in the present study generated from electrophysiological recordings from females with synthetic, racemic 6-methylnonan-3-one supports previously observed female response to female-produced compounds (Jewett, 1993; Bjostad et al., 1995). The response appears to be genuine, and similar scenarios have been observed in lepidopteran species such as *Choristoneura fumiferana* (Birch, 1977; Light and Birch, 1979), and *Trichoplusia ni* (Palaniswamy and Seabrook, 1978). The behavioral significance of *H. occidentalis* females responding to their own sex pheromone has not been determined.

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