Chem. Senses 27: 343–352, 2002

Odor Discrimination using Insect Electroantennogram Responses from an Insect Antennal Array

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Abstract

Insects have a highly developed olfactory sensory system, mainly based in their antennae, for the detection and discrimination of volatile compounds in the environment. Electroantennogram (EAG) response profiles of five different insect species, *Drosophila melanogaster*, *Heliothis virescens*, *Helicoverpa zea*, *Ostrinia nubilalis* and *Microplitis croceipes*, showed different, species-specific EAG response spectra to 20 volatile compounds tested. The EAG response profiles were then reconstructed for each compound across the five insect species. Most of the compounds could be distinguished by comparing the response spectra. We then used a four-antenna array, called a Quadro-probe EAG, to see if we could discriminate among odorants based on the relative EAG amplitudes evoked when the probe was placed in plumes in a wind tunnel and in a field. Stable EAG responses could be simultaneously and independently recorded with four different insect antennae mounted on the Quadro-probe, and different volatile compounds could be distinguished in real time by comparing relative EAG responses with a combination of differently tuned insect antennae. Regardless of insect species or EAG amplitudes, antennae on the Quadro-probe maintained their responsiveness with higher than 1 peak/s of time resolution.

Introduction

The summed, DC potential responses of thousands of differentially and narrowly tuned olfactory receptor neurons on insect antenna comprise the whole antennal response to odor known as the electroantennogram or EAG (Schneider, 1957; Roelofs and Comeau, 1971). The EAG can register responses to wide varieties of odorants and although it has been successfully employed for pheromone identifications over the years, it is now increasingly being used for identifying host-odor volatiles important to behaviors such as attraction (Cork, 1994; Cossé *et al*., 1995; Blight *et al*., 1997; Honda *et al*., 1999). Its success in this application stems from its ability to respond to serially eluting volatiles from the gas chromatograph that represent widely varying chain-lengths, volatilities and functional groups (Arn *et al*., 1975).

Despite its broad response spectrum, there is some evidence that EAG response spectra to a range of odorants are somewhat species-specific (Smith and Menzel, 1989; Visser *et al*., 1996; Visser and Piron, 1997). Indeed, the specificity of male moth antennae to their species' pheromone components has been instrumented in most pheromone identifications. The EAGs from single male antenna have been successfully used outdoors and indoors to sample airborne odor plumes in order to monitor the relative concentrations and even plume structures of high concentrations of known pheromone odorants to which the selected EAG detector is tuned, and have shown the potential utility of using insect antennae as sensitive biosensors for known agents in the environment (Baker and Haynes, 1989; Sauer *et al*., 1992; Karg and Sauer, 1995; Pers and Minks, 1998). However, a single EAG signal cannot be used to discriminate one unknown volatile compound from another, or even between a known pheromone component and unknown plant volatiles, mainly due to the confounding effects caused by unknown concentration differences. Thus, for a wider variety of general odorants to which antennae are not specifically tuned, the lack of odor discriminability of an EAG from a single antennal type will obviate the EAG's utility as a biosensor for detection and location of the sources of known target odors in the environment.

Our goal in this study was to create a discriminating EAG biosensor by utilizing the subtle differences in the EAG responses of several antennae from different species of insects monitored simultaneously. We also wanted to make optimal use of the EAG's ability to respond to sharp peaks in concentration, such as those generated from the individual strands in an odor plume (Baker and Haynes, 1989), in order to optimize the sensitivity of our biosensor. The success of such a system is predicated on there actually being enough of a consistent difference in EAGs across species such that the differences can in fact be used for discrimination. Another requirement is that these differ-

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ences should also be maintained across a range of concentrations, thereby eliminating confounding effects of concentration on odor quality discrimination. Despite there having been countless studies over the years on EAG responses within a species, to our knowledge, there has been no systematic examination of EAG response profiles to non-pheromonal odorants across species in any single study to date. This study presents the results of such a systematic examination.

Materials and methods

Insects

Adults of six insect species, *Helicoverpa zea* (Lepidoptera: Noctuidae), *Heliothis virescens* (Lepidoptera: Noctuidae), *Ostrinia nubilalis* (Lepidoptera: Noctuidae), *Trichoplusia ni* (Lepidoptera: Noctuidae), *Microplitis croceipes* (Hymenoptera: Braconidae), and *Drosophila melanogaster* (Diptera: Drosophilidae), were used. Colonies of the four moth species were maintained in a rearing room (23°C, 14:10 h L:D). Virgin moths, 1–3 days old, were used for the experiments. Pupae of the parasitic wasp, *M. croceipes*, kindly provided by Dr J. Lewis (USDA, Tifton, GA), were maintained in a cage provided with sugar solution and water. Adult wasps that emerged were harvested daily, sexed and kept in separate cages until use; 1- to 5-day-old wasps were used for the experiments. A colony of *D. melanogaster* (wild type, *Oregon* strain) was provided by Ms G. Lephardt (Genetics Laboratory, Iowa State University, Ames, IA) and maintained in the rearing room. Healthy looking active fruit flies were selected and used for the experiments. All the adults were provided with water and sugar solution until use.

Test compounds

Twenty test compounds, *cis*-11-hexadecenal (*Z*11–16:Ald), *cis*-3-hexenol (*Z*3–6:OH), hexanoic acid, benzyl acetate, 2-methyl-5-nitroaniline, cyclohexanone, α-pinene, *cis*-nerolidol, *trans*-nerolidol, β-caryophyllene, β-ocimene, (*R*)-(+)-limonene, methyl jasmonate, 2-diisopropylaminoethanol, indole, 2,2-thiodiethanol, 1-heptanol, 1-octanol, 1-nonanol and 1-decanol (chemical and isomeric purity >99%), were either provided by Dr J.H. Tumlinson (USDA, Gainesville, FL) or purchased (Aldrich®). Each compound was diluted in hexane or in acetone (for 2-methyl-5-nitroaniline and 2-diisopropylaminoethanol) to give 10 or 100 μg/μl solution. A piece of filter paper (6 × 32 mm) impregnated with 10 μl of test solution was inserted into a glass Pasteur pipette after the solvent evaporated and used as a stimulus cartridge. The cartridge was freshly made each time and the large end was capped with aluminum foil until use.

Comparison of EAG profiles across five insect species

To investigate the concept that the EAGs from a small array of antennal types can display different profiles to different odorants, EAG responses to 20 different compounds were recorded in five insect species: male *H. zea*; male *H. virescens*; male *O. nubilalis*; female *M. croceipes*; and male *D. melanogaster*. Antennae still attached to the whole body were used for the EAG recordings without cutting the antennal tips. The insect's body was restrained on a block of wax using small U-shaped metal wires. Antennae were further fixed with thinner copper wires onto the wax block. Using micromanipulators, a fine-tip micro glass electrode, filled with 0.5 M KCl solution, was inserted into a compound eye and served as a reference electrode. Another fine-tip micro glass electrode was inserted through the cuticle of the terminal segment of the antenna and served as a recording electrode. The antenna was positioned in the middle of humidified, main air stream for stimulation. EAG signals from the antenna were amplified with a headstage preamplifier (Syntech®, The Netherlands) and further processed with a PC-based signal processing system (Syntech®, The Netherlands).

Odor stimuli were applied with a main airflow (1.2 l/min) blowing onto the antennae through a glass tube (8 mm diameter). The outlet of the glass tube was positioned ~3 cm from the antennae. The tip of a Pasteur pipette odor cartridge was inserted into a small hole (3 mm diameter) on the main airflow tube, 15 cm from the outlet. Odor stimulation was administrated by injecting a puff of purified air (0.2 s at 10 ml/s airflow) through an odor cartridge using an electronically controlled stimulus flow controller (SFC-2, Syntech®, The Netherlands).

Four channel Quadro-probe EAG recording

In order to discriminately and sensitively sample airborne odor plumes, a portable, Quadro-probe EAG recording system (Figure 1) for simultaneous EAG recordings from four different antennae was designed in conjunction with Syntech® (The Netherlands). The probe had a common reference electrode and four independent recording electrodes. Excised antennae were used for the Quadro-probe EAG recording. Four different antennae (this time chosen from male and female *H. zea*, male and female *T. ni*, male *H. virescens*, male and female *M. croceipes*, and male *O. nubilalis*) were placed between the reference and the recording electrodes and an electroconductive gel (Spectra 360, Parker Laboratories Inc., USA) was used for the maintenance of electrical contact between the antennae and the electrodes. The EAG signals from the Quadro-probe were amplified and monitored with a four-channel portable amplifier unit (Syntech®, The Netherlands) and stored on a four-channel FM magnetic tape data storage unit as analog signals (Vetter Co. Inc., USA). The stored signals were further analyzed with PC-based data processing software designed for four-channel EAG recording analysis (Syntech®, The Netherlands).

Quadro-probe EAG recordings were made in point source

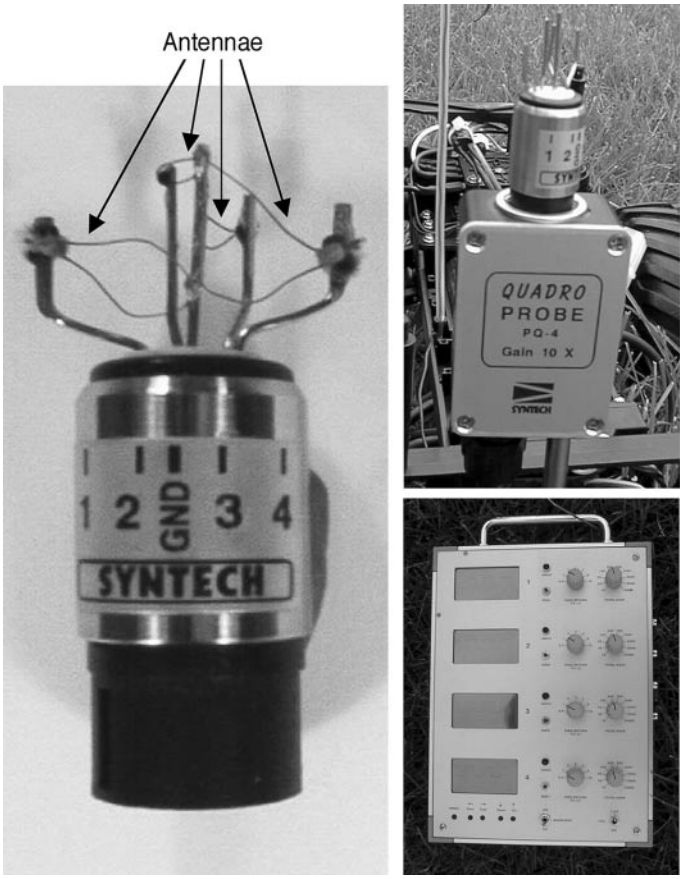

Figure 1 A portable Quadro-probe EAG recording system, enabling simultaneous, real-time EAG recordings from four different insect antennae on site. Electroantennogram signals from the four different antennae mounted on the Quadro-probe stage (left) are conditioned and amplified with a headstage pre-amplifier (top right) and further amplified and monitored with a main amplifier (bottom right).

odor plumes in a wind tunnel (1 × 1 × 2.4 m) and in a field. The Quadro-probe antennal preparation was positioned in the middle of the tunnel ~30 cm from the tunnel's downwind end. Wind speed was maintained at 30 cm/s. Odor plumes were generated from the headspaces of glass vials containing 10 μg/μl of different odorant solutions in hexane, placed 1.8 m upwind of the Quadro-probe sensor. Manual puffs through odor cartridges positioned at the center of the upwind end of the tunnel were also employed in order to obtain readings from the Quadro-probe EAG. The large end of the pipette was connected by means of a Teflon tube to a 10 ml glass syringe outside the tunnel. A 2 ml of puff was manually given through the syringe for stimulation. Field experiments were carried out at a parking lot outside the Science II building of Iowa State University. Odor presentation in the field was similar to wind tunnel experiments, except that the odor sources were placed 3 m from the Quadro-probe sensor. Wind speed was within 2 m/s and temperature was ~20°C.

Cluster analysis

Cluster analysis of the EAG response spectra was carried out to compare the proximity of EAG responses between different compounds and also between different insect species. Statistica® v. 5.1, a software package for statistic analysis (StatSoft Inc.), was used for the cluster analysis. Average normalized EAG amplitudes were used for the analysis and the tree clustering was made by complete linkage with Euclidean distances.

Results

All EAG responses elicited by a single puff in the five insect species examined showed a typical waveform, with rapid depolarization followed by a slower recovery phase, typically lasting a few seconds, back to the standing potential. There were no noticeable differences in the EAG response waveforms between typical single EAG recordings and Quadro-probe recordings for the single puff stimulation given by Pasteur pipette odor cartridge.

EAG response profiles of five different species in response to 20 different odorants showed that each species exhibited a different EAG response profile to the odorants (Figure 2). Among the compounds tested, *Z*3–6:OH and cyclohexanone elicited consistently high EAG responses across the antennae of all five species tested. Although the volatility was very low, an explosive compound, 2-methyl-5-nitroaniline, elicited EAG responses in two species, *H. zea* and *M. croceipes*, while other species did not respond. Two other anthropogenic compounds, 2-diisopropylaminoethanol and 2,2-thiodiethanol, also elicited EAG responses in all the species tested.

When these EAG responses were displayed according to compound, not species, the EAG profiles were often quite distinct from this five-species array (Figures 3–5). Even though the EAG response profiles of two relatively closely related species, *H. virescens* and *H. zea*, were quite similar, their relative responses were useful in discriminating among odorants when placed in the five-species array (e.g. Figure 3). Only a few closely related compounds, such as 1-heptanol and 1-octanol (and also 1-nonanol and 1-decanol), evoked nearly identical EAG response profiles from this array, but even these two shorter-chain alcohols can be distinguished from the two slightly longer-chain alcohols (Figure 6).

The cluster analysis across different compounds well shows that chemically different compounds could be discriminated by comparing EAG response spectra (Figure 7, top). The cluster analysis across different insect species also showed that the EAG response spectra were closely related to evolutionary proximity among the species (Figure 7, bottom). For example, two closely related noctuid moths, *H. virescens* and *H. zea*, showed the closest EAG response profiles and a dipteran species, *D. melanogaster*, showed the greatest distance from other for moth species in the EAG response spectra.

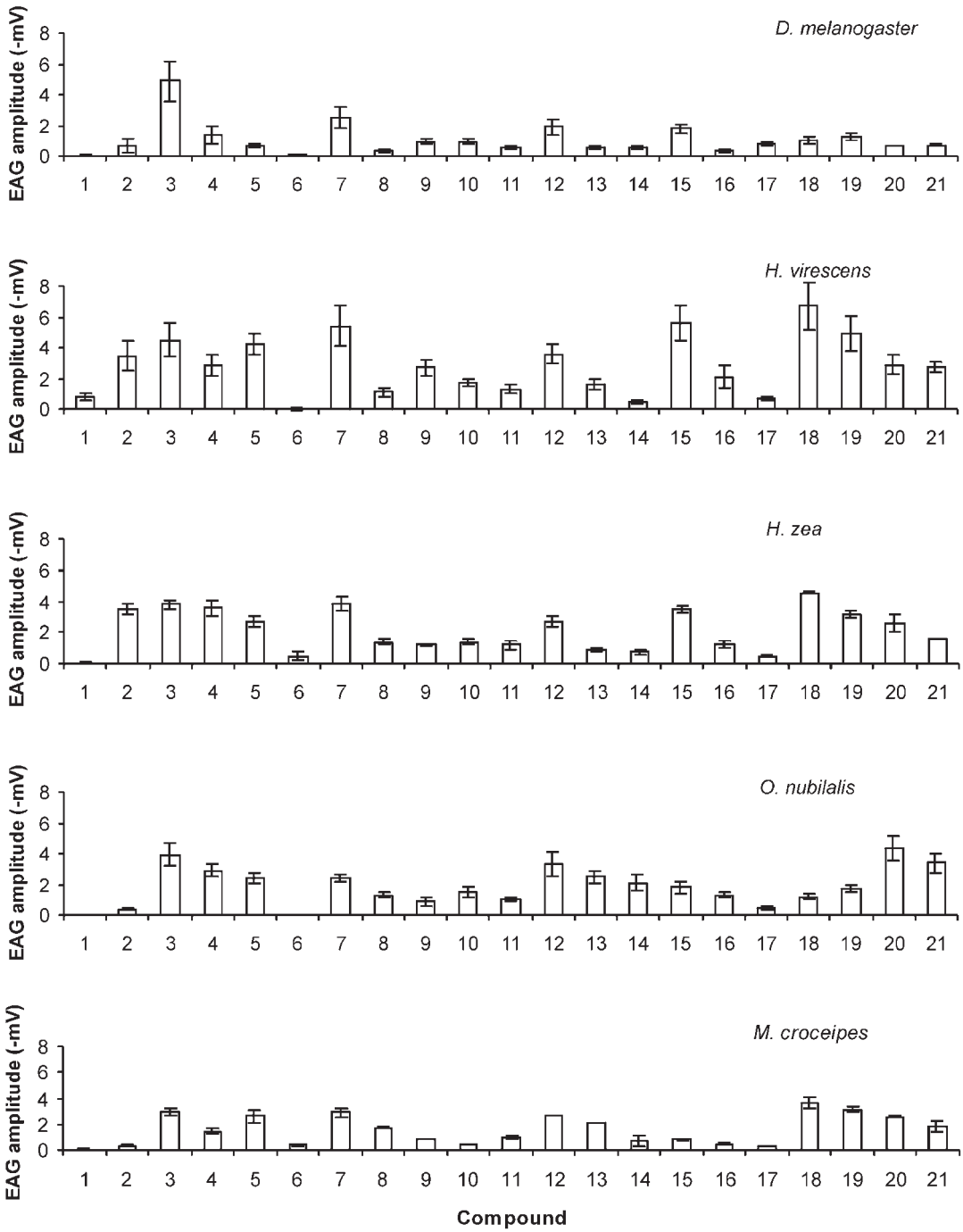

Figure 2 EAG response profiles of five different insect species (male *D. melanogaster*, male *H. virescens*, male *H. zea*, male *O. nubilalis*, female *M. croceipes*) to 20 different volatile compounds (mean ± SE, $n = 3$–19). Stimuli: (1) control blank; (2) *Z*11–16:Ald; (3) *Z*3–6:OH; (4) hexanoic acid; (5) benzyl acetate; (6) 2-methyl-5-nitroaniline; (7) cyclohexanone; (8) α-pinene; (9) *cis*-nerolidol; (10) *trans*-nerolidol;(11). β-caryophyllene; (12) β-ocimene; (13) (*R*)-(+)-limonene; (14) methyl jasmonate; (15) 2-diisopropylaminoethanol; (16) indole; (17) 2,2-thiodiethanol; (18) 1-heptanol; (19) 1-octanol; (20) 1-nonanol; (21) 1-decanol.

When EAG responses were simultaneously recorded from four different insect antennae mounted on the Quadroprobe EAG in a plume, stable EAG responses could be recorded with our system (Figures 8 and 9). The EAG responses were repeatedly generated by contact with the odor strands in the plume (Figures 8 and 9) and the relative ratios of EAG responses among the species antennae were maintained at a relatively constant level (Table 1). Each EAG response had a fast recovery time (<1 s; Figures 8 and 9) and the responsiveness was maintained throughout the entire stimulation period.

The high degree of synchrony of the depolarization

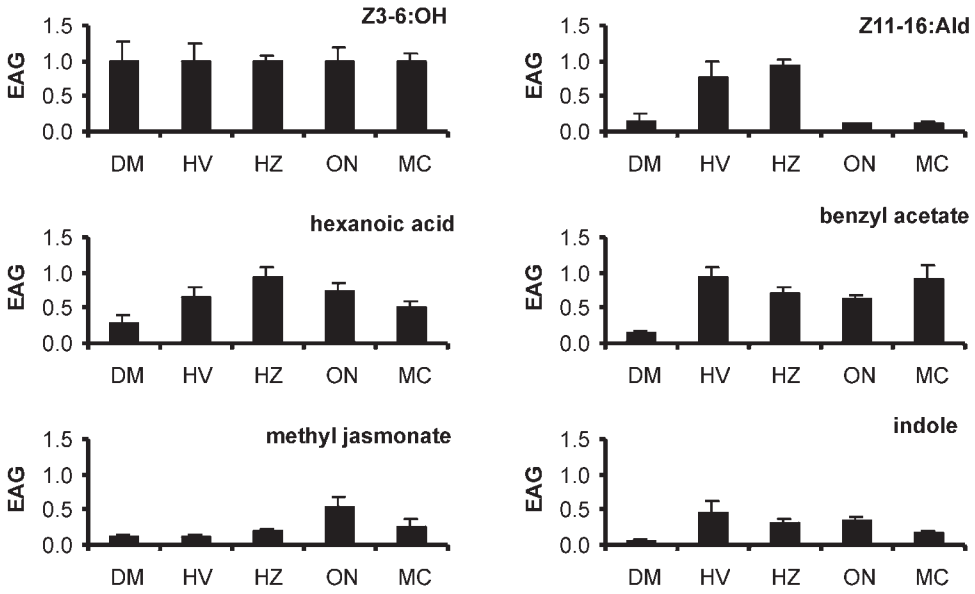

Figure 3 EAG response spectra across five different insect species to six different categories of volatile compounds (mean ± SE, $n = 3–19$) (*Z*3–6:OH, green leaf volatile alcohol; *Z*11–16:Ald, sex pheromone component of *H. zea* and *H. virescens*; hexanoic acid, aliphatic carboxylic acid; benzyl acetate, aromatic compound; methyl jasmonate, commonly occurring plant volatile; indole, nitrogen-containing animal odor). EAG responses were normalized against average EAG response to *Z*3–6:OH in each insect species (i.e. normalized EAG response to *Z*3-hexenol is 1). DM, male *D. melanogaster*; HV, male *H. virescens*; HZ, male *H. zea*; ON, male *O. nubilalis*; MC, female *M. croceipes*.

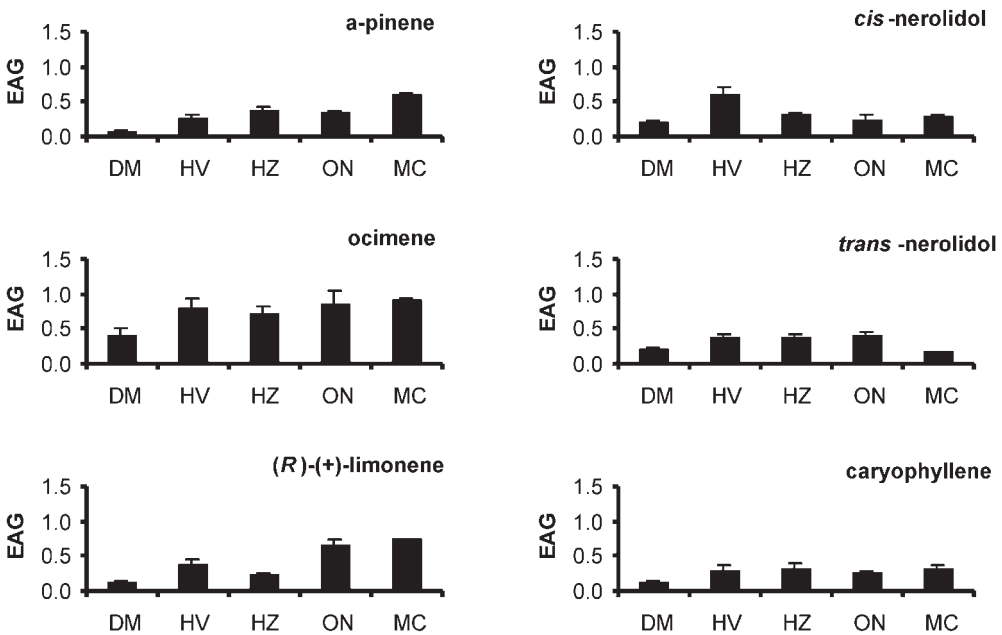

Figure 4 EAG response spectra across five different insect species to three different monoterpenes (left) and three different sesquiterpenes (right) (mean ± SE, $n = 3$). EAG responses were normalized against average EAG response to *Z*3–6:OH in each insect species (i.e. normalized EAG response to *Z*3-hexenol is 1). DM, male *D. melanogaster*; HV, male *H. virescens*; HZ, male *H. zea*; ON, male *O. nubilalis*; MC, female *M. croceipes*.

between antennae as they were contacted by closely arriving filaments is apparent in an expanded temporal display of the signals (Figures 8 and 9). Such synchrony would facilitate the real-time assessment of odorant quality by computer algorithms comparing EAG peaks at any instant across the Quadro-probe array. The variability in the ratios of any of the major EAG peaks across the four antennae was similar to the average, indicating the utility of using contacts with even single strands of odor for discrimination of odorants.

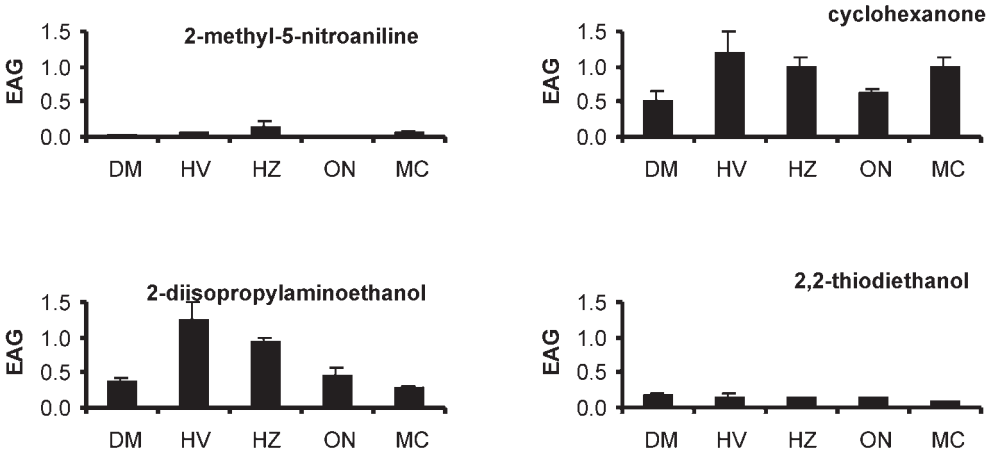

Figure 5 EAG response spectra across five different insect species to four different anthropogenic compounds (mean ± SE, $n = 3$–12). EAG responses were normalized against average EAG response to *Z*3–6:OH in each insect species (i.e. normalized EAG response to *Z*3-hexenol is 1). DM, male *D. melanogaster*; HV, male *H. virescens*; HZ, male *H. zea*; ON, male *O. nubilalis*; MC, female *M. croceipes*.

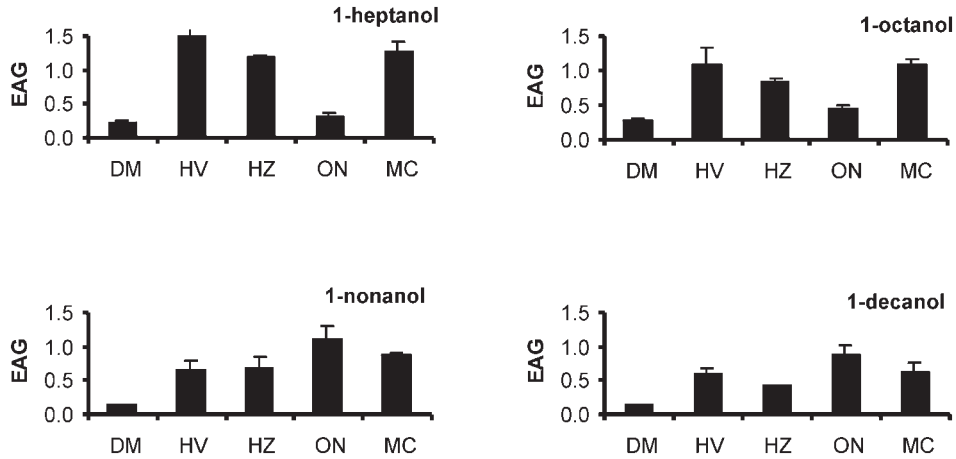

Figure 6 EAG response spectra across five different insect species to four different aliphatic alcohols (mean ± SE, $n = 3$). EAG responses were normalized against average EAG response to *Z*3–6:OH in each insect species (i.e. normalized EAG response to *Z*3-hexenol is 1). DM, male *D. melanogaster*; HV, male *H. virescens*; HZ, male *H. zea*; ON, male *O. nubilalis*; MC, female *M. croceipes*.

Discussion

The present study demonstrates the basic concept of odor discrimination using a small array of insect antennae that have different EAG response spectra to a wide range of compounds. With EAG response-spectra across only five different species, we were able to discriminate most of the 20 different compounds tested. Several successful studies have been conducted that have measured EAG responses to single, known odorants under field conditions (Baker and Haynes, 1989; Sauer *et al.*, 1992; Karg and Sauer, 1995; Milli *et al.*, 1997; Pers and Minks, 1998; Schütz *et al.*, 1999). These studies have shown that it is possible to measure the relative concentrations and fluctuations of known pheromone components in ambient air on site in real time. The Quadro-probe EAG recording system developed in the present study showed stable EAG recording ability to a repeated stimulation by odor strands in the plume, as well as to single puffs under laboratory conditions. Responses to odor plumes have also been successfully made by the Quadro-probe under field conditions (K.C. Park *et al.*, unpublished data).

As previously shown (Baker and Haynes, 1989) and again here, the ability of an insect antenna to respond quickly to peak concentration in the strands in a plume makes it a much more sensitive system than slower-responding artificial sensors whose polymers can only take a reading of a time-averaged mean, not the peak, concentration (Walt *et al.*, 1998; Kasai *et al.*, 1999). The present study, like that of Baker and Haynes, shows that an insect antenna placed in a plume is able to respond to the individual filaments in a point-source-generated plume. When the antennae were stimulated by repeated contact with filaments in a plume in the wind tunnel, the antennae were able to register two or three depolarizations per second. Therefore, the use of insect

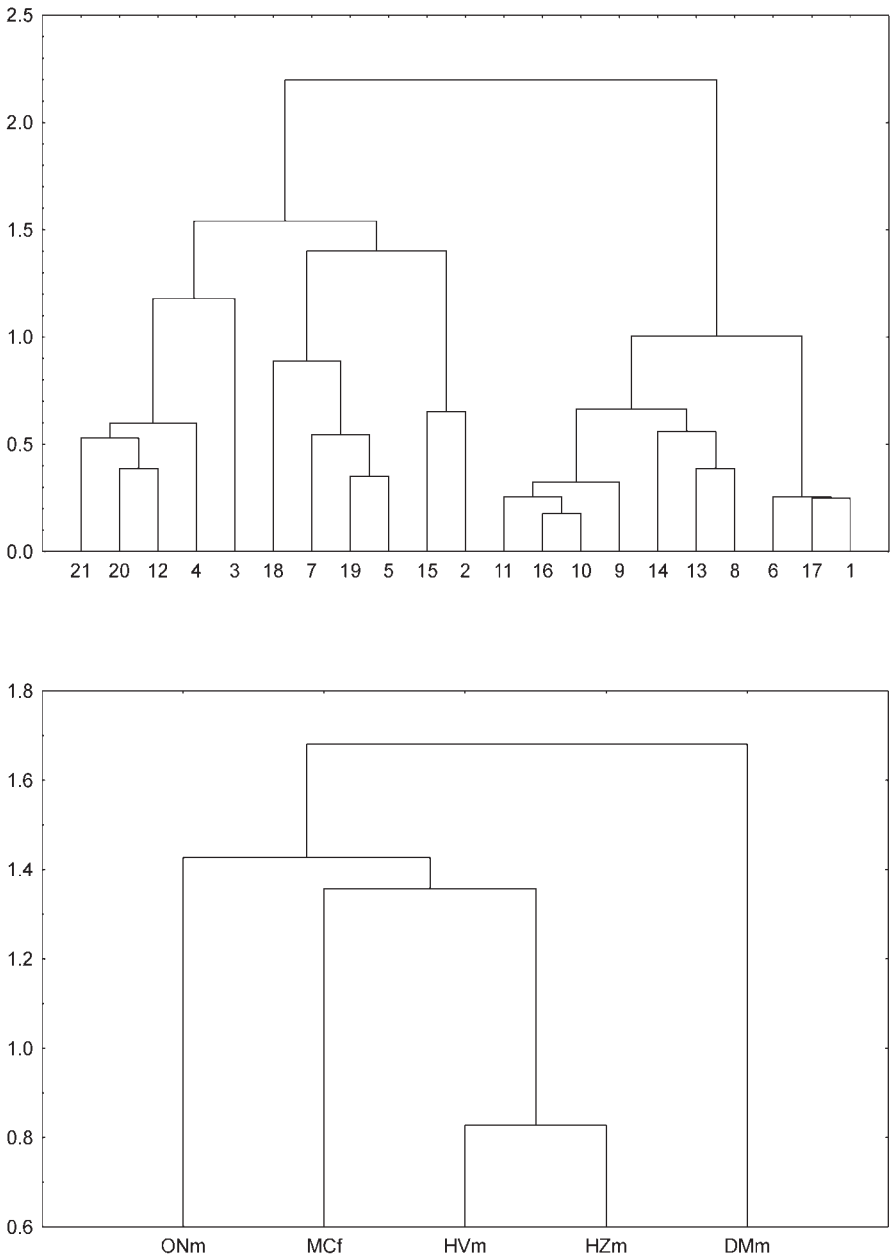

Figure 7 Cluster analysis of EAG response spectra for different compounds. Clustering for different compounds was obtained across five different insect species (top: MCf, HZm, HVm, Onm) or across four different insect species (bottom). Average normalized EAG responses to *Z*3–6:OH were used for the clustering (complete linkage and Euclidean distances). MCf, female *M. croceipes*; HZm, male *H. zea*; HVm, male *H. virescens*; Onm, male *O. nubilalis*; HZf, female *H. zea*; TNm, male *T. ni*.

antennae as an odor detector could be one of the most sensitive techniques for the detection of volatile compounds, especially when they are present at low concentrations.

Recently, several attempts have been made to use single insect antennae as olfactory biosensors (Kuwana and Shimoyama, 1998; Schöning *et al*., 1998; Kuwana *et al*., 1999; Schroth *et al*., 1999; Schütz *et al*., 1997, 1999). Although they have showed sensitivity for the detection of known volatile compounds of interest, such an apparatus with a single, tuned detector lacks the ability to discriminate the compound of interest from unknowns due to the confounding effects of concentration. Antennae have been used in highly simplified odor environments to detect specific volatile compounds, for instance those emanating from unhealthy potatoes (Schütz *et al*., 1999), measuring cross-sectional relative concentration differences across plumes (Miller and Roelofs, 1978), measuring the pattern of pulsed pheromone emissions from female arctiid moths (Conner *et al*., 1980), as well as monitoring the structure of pheromone plumes (Baker *et al*., 1985; Baker and Haynes, 1989). Now

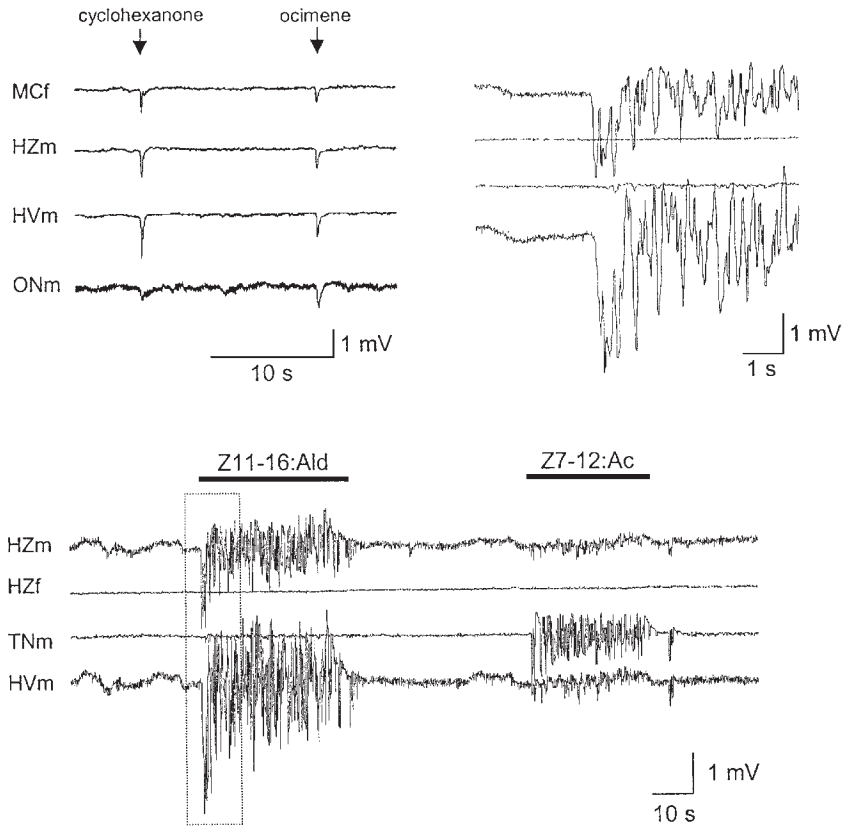

Figure 8 Simultaneously recorded EAG responses elicited by four different antennae mounted on a Quadro-probe in a wind tunnel. Top left: EAG responses to single puffs of two different stimuli. Bottom: EAG responses to two separate continuous odor plumes. Top right shows the details of the responses in the dotted box. MCf, female *M. croceipes*; HZm, male *H. zea*; HVm, male *H. virescens*; Onm, male *O. nubilalis*; HZf, female *H. zea*; TNm, male *T. ni*.

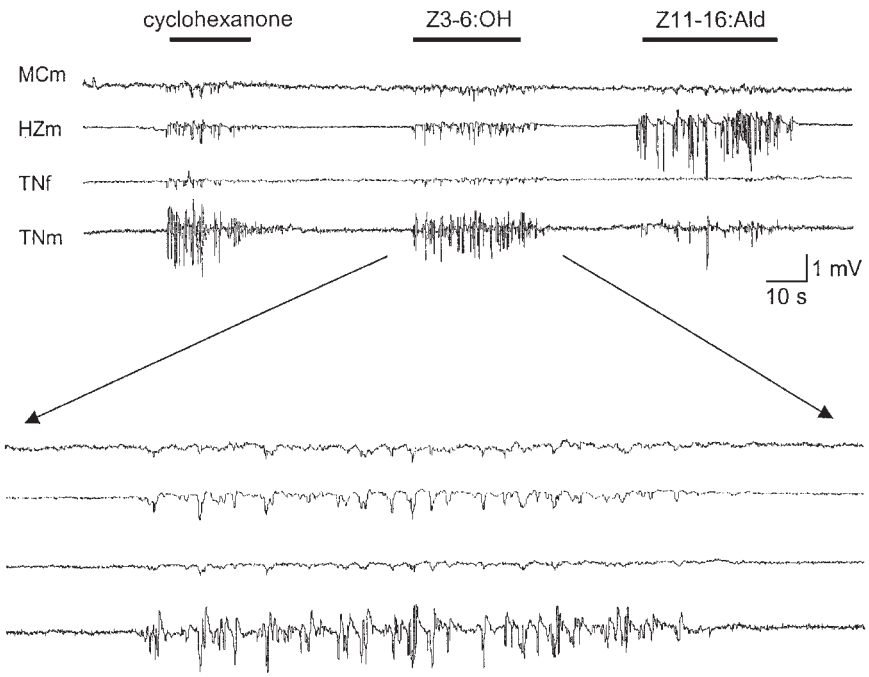

Figure 9 Simultaneously recorded EAG responses elicited by four different antennae mounted on a Quadro-probe in a field. Three different stimuli (cyclohexanone, *Z*3–6:OH and *Z*11–16:Ald) were consecutively applied from ~3 m upwind from the Quadro-probe antennal preparation. MCm, male *M. croceipes*; HZm, male *H. zea*; TNf, female *T. ni*; TNm, male *T. ni*.

Table 1. EAG responses of four different antennae mounted on a quadro-probe system to three different stimuli in plumes in field (mean ± SE)

EAG[a]	Stimulus[b]	Antennae[c]				*n*
		MCm	HZm	TNf	TNm	
Absolute EAG (–mV)	Cyclohexanone	0.21 ± 0.034	0.41 ± 0.057	0.12 ± 0.030	1.45 ± 0.100	15
	Z3-6:OH	0.17 ± 0.028	0.42 ± 0.033	0.09 ± 0.016	0.91 ± 0.063	27
	Z11-16:Ald	0.04 ± 0.013	1.37 ± 0.071	0	0.24 ± 0.058	38
Normalized EAG	Cyclohexanone	0.47 ± 0.052	1 ± 0	0.29 ± 0.064	4.18 ± 0.518	15
	Z3-6:OH	0.37 ± 0.053	1 ± 0	0.20 ± 0.038	2.38 ± 0.197	27
	Z11-16:Ald	0.02 ± 0.007	1 ± 0	0	0.14 ± 0.028	38

[a]Absolute amplitudes of major EAG peaks were measured in a quadro-probe recording in field (see Figure 9). EAG responses elicited at the same time from the four antennae were subsequently normalized against male *H. zea*.

[b]Hexane solution of each compound was freshly loaded onto a piece of filter paper and placed ~3 m upwind from the quadro-probe antennal preparation (cyclohexanone and Z3-6:OH: 1 mg; Z11-16:Ald: 100 μg).

[c]MCm: male *M. croceipes*; HZm: male *H. zea*; TNf: female *T. ni*; TNm: male *T. ni*.

we have shown the potential for an array of antennae to provide discrimination as well as sensitivity. The present study was initialized to develop an odor detector for the location of low-level volatile compounds being emanated from explosive compounds such as DNT and TNT, and some other toxic chemicals such as 2-diisopropylaminoethanol and 2,2-thiodiethanol. Our results are promising in this regard because they have demonstrated that these anthropogenic compounds can be detected and discriminated using a simple array of insect antennae.

The present study demonstrates that different odor compounds can be distinguished by comparing the relative EAG responses of the antennae from different insect species using the multiple antennae recording technique. Some compounds having quite similar structures (such as 1-heptanol and 1-octanol, or 1-nonanol and 1-decanol) did not appear to be easily distinguished by using this technique with the antennal array from the five different species chosen for the present study. However, depending on the application, such compounds could likely be discriminated by different antennae from species other than these five that might have more disparate response spectra to those compounds.

The cluster analysis indicates that compounds may be discriminated using this technique and seems to sort out to some degree according to chemical class. Applying cluster analysis to the single puff results shows the potential of using such analysis in real time on the plume readings from the Quadro-probe array. It would be possible to use neural network analysis to create an initial template memory of the odor to be searched for, then use the array to search for it.

Many studies have indicated that there is a variation in the EAG responses between different individuals (Park and Hardie, 1998) and the normalization of the EAG responses has been widely used to exclude this individual variation for evaluating the EAG responses (Park and Hardie, 1998). However, recent studies have suggested that such individual variation can be minimized by improving the EAG recording technique (Ayer and Carson, 1992; Park *et al*., 2000). Once a stable and reproducible EAG recording technique is established, normalization is no longer essential for compensating between variations among individuals in EAG responses. The limited lifetime of the preparation has also been an obstacle in the use of living tissues as biosensors (Kuwana and Shimoyama, 1998; Kuwana *et al*., 1999; Schroth *et al*., 1999; Schütz *et al*., 1999). Although different recording protocols showed considerable variations in the lifetime of EAG preparation, usually EAG preparations do not last >2 h (Hardie *et al*., 1994; Visser *et al*., 1996; Visser and Piron, 1997) and often <30 min (Giessen *et al*., 1994). However, recent advances have shown that the lifetime of an EAG preparation can be considerably extended—for >8 h (Park and Hardie, 1998; Park *et al*., 2000). Further extending EAG longevity would, of course, increase the usefulness of insect antennae as olfactory biosensors and research needs to continue along these limits.

Acknowledgements

The authors wish to thank Dr J. Lewis for providing the wasps and Ms G. Lephardt for the fruit flies, and also wish to thank Ms J. Harris for maintaining the colony. Dr Jan van der Pers is thanked for the Quadro-probe development. This study was funded by the Defense Advanced Research Projects Agency (DARPA) in the United States.

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Accepted January 17, 2002