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Detection and Discrimination of Mixed Odor Strands in Overlapping Plumes Using an Insect-Antenna-Based Chemosensor System

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Abstract Olfactory signals, a major means of communication in insects, travel in the form of turbulent odor plumes. In terrestrial environments, an odor blend emitted from a single point source exists in every strand of the plume, whereas, in confluent plumes from two different odor sources, the strands have some chance of being coincident and comprising a new third odor in those strands. Insects have the ability to detect and interpret necessary olfactory information from individual filamentous odor strands in complex multifilament odor plumes. However, behaviorists have had no way to measure the stimulus situations they are presenting to their temporally acute insect subjects when performing Y-tube olfactometer or confluent pheromone plume wind tunnel assays. We have successfully measured the degree of plume-strand mixing in confluent plumes in a wind tunnel by using a multichannel insect-antenna-based chemosensor. A PC-based computer algorithm to analyze antennal signals from the probe portion of the system performed real-time signal processing and, following a short training session, classified individual odorant/mixture strands at sub-second temporal resolution and a few tens of millimeters of spatial resolution. In our studies, the chemosensor classified a higher frequency of strands of two different odorants emitted from two closely spaced filter papers as being "mixed" when the sources were located only 1 or 2 cm apart than when the sources were 5 or 10 cm apart. These experiments demonstrate the chemosensor's potential to be used for measuring odor stimulus situations in more complex multiple-plume environments.

Keywords Electroantennogram · Insect antennae · Odor detection · Odor strand · Odor plume · Tissue-based chemosensor · Volatile detection · Odor strand detection · Odor strand discrimination

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Introduction

Insects have demonstrated in behavioral experiments that use experimentally pulsed pheromone puffs that they are capable of discriminating pheromone plume strands of incompletely mixed components that are separated by only 1 mm from each other or equivalently by 1/1,000 of a second (Baker et al. 1998). However, researchers involved in presenting odors to insects that emanate from natural point source plumes in more complex contexts have not had the ability to measure the sub-second odor stimulus conditions against background odor plumes that may be experienced by insects in determining their behavioral responses. The ability to measure on a sub-second basis the degree of mixing of, for instance, plume strands of plant volatiles with those of synthetic or natural pheromone components or alternatively two different species' pheromone plumes would provide a quantitative basis for understanding olfactory temporal acuity that affects behavioral responses. The high time resolution of insect antennae to various odorants and the well-established signal recording technique from insect antennae such as electroantennogram (EAG) and

single-cell recording previously had given researchers the idea of using insect antennae as olfactory chemosensors to detect various volatile compounds of interest and ascertain fine-scale plume structure features (Baker and Haynes 1989; Sauer et al. 1992; Huotari and Mela 1996; Schöning et al. 1998; van der Pers and Minks 1998; Park et al. 2002).

In natural environments, point source odor plumes with their fine-scale plume-strand structures do not exist in solitude. The strands of a plume from one source can intermingle with those from another. The degree of confluent plume-strand coincidence or separation can have significant effects on behavior (Vickers and Baker 1992; Baker et al. 1998) or on olfactory receptor neuron (ORN) activity (Nikonov and Leal 2002; Ochieng and Baker 2002). We have had, to this point, however, no way of measuring the stimulus situations actually experienced by insects under various odor plume presentation regimes that affect their subsequent responses.

With single-antenna EAG recordings, odor discrimination cannot be performed due to the confounding effects of concentration and chemical composition. However, in recent studies, we showed that a four-channel insect-antenna-based chemosensor system that uses an array of slightly differentially tuned insect antennae, named the "Quadro-probe," could be used to discriminate various odors on a strand-by-strand basis (Park et al. 2002; Hetling et al. 2003; Myrick et al. 2005). The Quadro-probe has sub-second temporal resolution of individual odor strands (and a few millimeters of spatial resolution) that enables detailed odor-plume-strand structure and composition to be performed. A dedicated analysis software package (Hetling et al. 2003; Myrick et al. 2005) that can be trained to recognize EAG patterns across particular arrays of different insect antennae in response to different odors was also developed. The system has enabled the performance of real-time high-throughput odorant signal classification and monitoring. In the present study, the Quadro-probe system was used to investigate the degree of mixing of overlapping odor plume strands emitted from dual point sources, which provides a proof-of-concept foundation for investigating more complex multiple-point-source strand discrimination.

Our hypotheses were: first, that we should be able to differentiate between strands sheared from a single-source two-odorant mixture and those emitted from two closely spaced separate odorant sources; second, that there should be a greater degree of plume-strand coincidence downwind from two overlapping plumes of different odorants when the upwind sources are closer together than when the sources are more widely spaced. The ability to confirm or falsify these hypotheses and the ability to measure the degrees of plume-strand mixing have potential impact on various insect chemical ecology studies under natural conditions where plant volatiles may interact to influence olfaction and behavioral response to pheromones (Ochieng and Baker 2002) or where pheromone plumes of two species might interfere with males' behavioral responses (Witzgall and Priesner 1991; Liu and Haynes 1992; Vickers and Baker 1997; Nikonov and Leal 2002).

Methods and Materials

Insects One- to 3-day-old virgin male moths of the corn earworm, *Helicoverpa zea*, and the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae), were used. Laboratory colonies of these species were maintained on artificial diet under controlled conditions (14:10 h L:D, 23°C, 60% relative humidity) in a rearing room. Male pupae were kept in separate containers, and emerged adults were collected daily and provided with 8% sucrose solution until use.

Test Chemicals Two compounds, (*Z*)-11-hexadecenal (Z11-16:Ald) and (*Z*)-7-dodecenyl acetate (Z7-12:Ac), were used in experiments 1 and 2. These compounds are the major sex pheromone components of *H. zea* and *T. ni*, respectively. Each compound (Bedoukian, >99% chemical and isomeric purity) was diluted in hexane to give a 1-μg/μl solution. A 1:1 mixture of these two compounds also was prepared at the same concentration. Filter papers (Whatman no. 1) cut to 8×40 mm were impregnated with the test solutions containing 10 μg of one of the three odors (Z11-16:Ald, Z7-12:Ac, or the 1:1 blend of the two compounds) and were then used to produce odor plumes in our wind tunnel. Each filter paper dispenser was used for less than 1 h and discarded. In experiment 3, a plant-related compound, citronellal (Acros Organics, 93% pure) at a dose of 100 μg per filter paper, was used as a second odorant to accompany the first odorant, Z11-16:Ald, at 10 μg per filter paper. A blend of these two compounds at these two doses emitted from a single filter paper comprised the blend, i.e., the third odor.

Wind Tunnel Experiments were carried out by using a Plexiglas® wind tunnel (1×1×2.5 m). Airflow was created by a large fan system, filtered with activated charcoal filter layers, and sent through a buffer area of three layers of fine mesh nets. An exhaust suction tube (50-cm diameter) was located at the downwind end of the tunnel to scavenge odor-bearing air from the plumes out of the wind tunnel room. The rest of the air from the tunnel was re-circulated through the room. Airflow rate was controlled by using a variable AC transformer to change electrical current for the fan system. A constant wind speed of 50 cm/s was maintained inside the tunnel, and the temperature was maintained at 23°C during experiments.

Experiment 1

Experimental Setup Two male *T. ni* and two male *H. zea* antennae were excised with micro-scissors from the heads and placed between stainless steel reference and recording electrodes to create the four input channels of the Quadro-probe. The bases of the four excised antennae were placed individually on each of the four reference electrodes, and their distal ends were brought into contact with the four different recording electrodes after clipping off a few terminal antennal segments. Electrical connections between the antennae and metal electrodes were secured with an electroconductive gel (Spectra 360, Parker Laboratories Inc., USA). EAG signals from the Quadro-probe were acquired with a headstage amplifier, amplified further with a main amplifier (Syntech, The Netherlands) and then processed and stored on a laptop PC. The Quadro-probe antennal preparation was placed within the time-averaged boundaries of the plume(s) in the middle of the tunnel at a downwind distance of 1.5 m during training sessions and at 1.5, 1.0, or 0.5 m downwind of the filter paper sources during various test situations. The filter paper dispensers were affixed to paper clips hung from metal stands 50 cm above the tunnel floor and 30 cm from the upwind end of the tunnel. Before the beginning of trials each day, a burning incense stick was placed at the upwind position where the stimulus dispenser was to be located. The plume of incense smoke was observed to ensure that the plume strands would be contacting the Quadro-probe antennal preparations.

Analysis Software A computer algorithm (Myrick et al. 2005) was used to acquire, process, and analyze four-channel EAG signals from the Quadro-probe; this system was run on a Labview-based platform (National Instruments, USA). The program carried out noise filtration, EAG signal identification and classification, and reporting of results. The classifier used a supervised *k*-nearest-neighbor (*k*-NN) procedure that was trained at the start of the experiment to recognize strands as being either Z7-12:Ac, Z11-16:Ald, or a 1:1 blend of the two compounds emitted from a single filter paper source. EAG "events" across the array were synchronized according to their occurrence within a 160-ms window. For instance, when a significant EAG depolarization (measured from trough to peak) having the greatest amplitude occurred on channel 1, the program created a 160-ms swath to look down onto the other channels to quantify the amplitudes of any other significant EAG events occurring on the other three channels.

Training and Classification with Single-Odor Plumes The session started with a period of training of the algorithm to three pheromone odor sources and clean air by subjecting the antennal array to plume strands from filter papers 1.5 m upwind that were dosed with 10 μg of test compounds (blank, Z11-16:Ald, Z7-12:Ac, and the 1:1 blend of Z11-16:Ald and Z7-12:Ac) for either a 20- or a 60-s period at a distance of 1.5 m downwind of the odorant source. These training periods were then used as criteria for odor strand classification during the subsequent test periods by the algorithm. We also tried a 60-s training period to see if classification accuracy would be improved. During the test periods, the antennal array was subjected to each test stimulus dispenser in random order for >120 s at 0.5, 1.0, and 1.5 m downwind of the odorant source, and the signals were digitized and stored. The number of replicates for each distance/stimulus combination is indicated in Table 1. For each test session, the signals were analyzed off-line by the computer algorithm for signal processing and odor classification.

Classification of Strands From Confluent Dual-Source Odor Plumes The degree of mixing of odor strands within overlapping plumes originating from two different closely positioned pheromone point sources was assessed by using the Quadro-probe system by detecting and classifying

Table 1 Comparison of performance of the EAG signal processing computer algorithm in positive signal identification (true positive) for odor strands of two sex pheromone components, (*Z*)-7-dodecenyl acetate and (*Z*)-11-hexadecenal, and their 1:1 mixture between two different training durations performed at 150 cm downwind and then tested at three different downwind distances

Distance (cm)	Stimuli	Percent correct recognition at training period of (mean±SE)		Number	*P* value
		20 s [total number of EAG signals]	60 s [total number of EAG signals]		
150	*Z*11-16:Ald	97.6±1.73 [113]	95.0±1.95 [127]	4	0.40
	*Z*7-12:Ac	100.0±0.00 [126]	100.0±0.00 [116]	4	N/A
	Mixture	89.5±4.75 [135]	92.6±1.53 [135]	4	0.39
100	*Z*11-16:Ald	92.8±5.58 [80]	93.8±3.27 [87]	3	0.65
	*Z*7-12:Ac	100.0±0.00 [79]	100.0±0.00 [114]	3	N/A
	Mixture	88.5±4.35 [100]	86.7±5.02 [79]	3	0.55
50	*Z*11-16:Ald	70.3±4.01 [161]	74.6±9.61 [138]	3	0.39
	*Z*7-12:Ac	100.0±0.00 [52]	97.0±3.03 [49]	3	0.30
	Mixture	100.0±0.00 [145]	98.1±1.20 [188]	3	0.08

Average number of EAG signals (depolarizations) in 20-s recording durations was 32.7±1.44 (mean±SE, N=84, range 7~73).

individual odor strands in the confluent plumes. Following the training session to the three separate odors from single sources, the same antennal preparation was used for the test sessions. The Z11-16:Ald and Z7-12:Ac odor sources were suspended on the rod with a 5-cm lateral separation between them at the upwind end. During this test period, signal acquisition and classification was performed for 120 s. Data were stored on a laptop PC for later analysis.

Experiments 2 and 3

Experimental Setup In the second and third experiments, live-moth preparations were used instead of excised antennae. Two male *T. ni* and two male *H. zea* were immobilized in tapered aerated plastic tubes and placed in a custom four-channel preamplifier (Fig. 1). With an electrolytically sharpened tungsten electrode inserted into the eye as a ground reference, the two antennae of each moth (with the antennal tips intact) were draped over the amplifier electrodes using Spectra 360 electroconductive gel to establish a connection to the amplifier.

Training Sessions Initial training sessions of approximately 100 s in plumes of each of three different odors, i.e., odorant 1 alone, odorant 2 alone, and a blend of odorants 1 and 2 at their initial dosages emitted from a single filter paper source, were conducted at the start of each experiment. EAG recordings were taken for an unpredetermined duration in the plume, but frequencies of classified odorant/odor "events" were standardized according to an events-per-second criterion.

Experiment 2 Once the training sessions had ceased, the Z11-16:Ald and Z7-12:Ac filter papers were suspended at the upwind end of the tunnel, beginning with a 1-cm lateral separation of the filter paper sources. After a period of sampling of approximately 100 s (ranging from 38 to 115 s) of strands from within a zone of confluence of the two plumes, the two sources were then separated by 2, then 5, and finally 10 cm. A second series of recordings was performed in reverse order, starting with the 10-cm source separation and working back to the 1-cm separation. Three experiments were performed, and because both increasing and decreasing the separation of the filter papers were known sources of potential variance, these experiments were broken into six blocks. Using Minitab ver. 14, two-way (distance of filter paper separation, block) analysis of variance (ANOVA) was performed on a general linear model of the strand frequencies of each odor. Tukey's pairwise comparisons were also performed on the model.

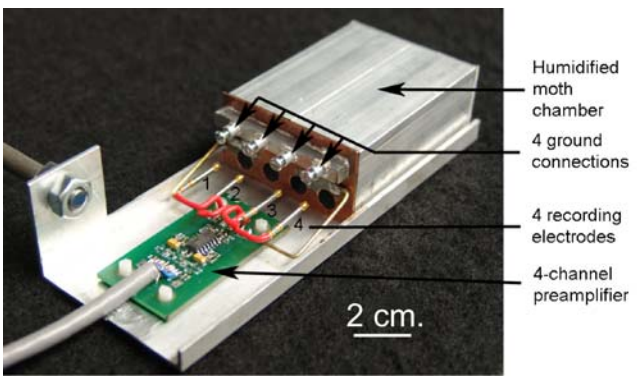

Fig. 1 Photograph of the whole-body preparation of the four-antenna Quadro-probe chemosensor probe using whole moths confined in plastic tubes within an aerated humidified aluminum chamber

Experiment 3 As in experiment 2, once the training sessions had ceased, the Z11-16:Ald and citronellal filter papers were suspended at the upwind end of the tunnel, beginning with a 1-cm lateral separation of the filter paper sources. After a period of sampling the odor strands within the confluent plumes, the two sources were separated by 2, then 5, and finally 10 cm, with data at all these distances being reported by the whole-body four-channel EAG preparation and stored on laptop PC for later analysis. Similar to experiment 2, a second series of recordings was performed in reverse order, starting with the 10-cm source separation and working back to the 1-cm separation. Two experiments were performed, and because both increasing and decreasing the separation of the filter papers were known sources of potential variance, these experiments were broken into four blocks. Using Minitab ver. 14, two-way (distance, block) ANOVA was performed on a general linear model of the strand frequencies of each odor. Tukey's pairwise comparisons also were performed on the model.

Classification of Odor Strands from Dual-Source Plumes In the second and third experiments that used live-moth preparations, the classifier made use of a Gaussian model for the probability density functions describing each odor. The preamplifier, seated in the whole-body apparatus, was connected to the recording system as described in Myrick et al. (2005). Data were filtered with a 2–15-Hz digital finite impulse response band pass filter prior to feature extraction. The feature type utilized in classification included time-synchronized EAG depolarization trough-to-peak voltages (TPV) only. When a depolarization occurred, trough-to-peak voltages (where a peak describes a *negative*-going depolarization) on different channels with (negative) peak times occurring within 160 ms were grouped to form a four-dimensional feature vector, called an event, with a feature corresponding to each channel. Missing data (when no peak was found on a channel) were filled with a value of 0. Any event (collection of four time-correlated trough-to-peak voltages) with no peaks larger than 100 μV was discarded as a simple strand detection procedure. The

trough-to-peak voltages in the remaining events were normalized by using the same constant so that the standard deviation of all the trough-to-peak values in the training set on all of the channels was unified. Note that this operation retains concentration information. The same value was used to keep noise levels relatively equal on each channel.

After collecting several hundred training events (100 s) for each odor, the probability densities of each odor were modeled by using a multidimensional Gaussian distribution. The Gaussian is able to model the unique correlation between the channels for each odor. The distribution was derived from a four-dimensional model, where variation along the covariance matrix eigenvector with the largest eigenvalue was ignored, so that the dimensionality of the distribution was reduced by 1. The component of any feature vector that points along this eigenvector is considered to be a measure of signal strength. The multidimensional Gaussian density (MDG) may be expressed by the following equation (Theodoridis and Koutroumbas 1999).

$$f(x) = \frac{1}{\sqrt{(2\pi)^d |\sum|}} \exp\left(-1/2(\mathbf{x}-\mu)^T \sum^{-1} (\mathbf{x}-\mu)\right)$$

where $\mathbf{x}$ is a vector of d (four) elements containing the feature values of an event (i.e., trough-to-peak measurements from multiple antennae); $\mathbf{\Sigma}$ is the sample covariance matrix of the trough-to-peak measurements for the odor, and $\boldsymbol{\mu}$ is the vector containing the feature means. The exponent of the MDG may be written as a summation in terms of the eigenvectors and eigenvalues of the covariance matrix. The sample covariance matrix may be expressed in terms of the eigenvectors and eigenvalues, known as spectral decomposition, or eigendecomposition, making its inverse simple to calculate (Johnson and Wichern 1992). We write it this way to explicitly remove dependence of the probability density in the direction of the eigenvector with the largest eigenvalue, so that the probability density near the mean is extrapolated through the origin. This estimates the density near the origin, where background and noise reside, since it cannot be measured directly. The problem of not having the ability to train on pure odor (that is only odor + background or background is available during training for an odor) is known as the imperfect teacher problem (Krishnan 2001) for mixture densities. It also makes the classification result independent of any prior knowledge of the distribution of signal strength for a particular odor. Let the index of the largest eigenvector and corresponding eigenvalue be $n$max. The reduced dimensionality MDG may be written

$$f(x) = \frac{1}{\sqrt{(2\pi)^{d-1} \prod_{n=1, n\neq n\max}^{d} \lambda_n}} \exp\left(-\frac{1}{2} \sum_{n=1, n\neq n\max}^{d} \frac{(\mathbf{e}_n \bullet (\mathbf{x}-\mu))^2}{\lambda_n}\right)$$

where $\mathbf{e}_n$ and λ_n are the nth eigenvector and eigenvalue of the sample covariance matrix for a particular odor, respectively. An example that illustrates the odor density functions superimposed on training data for two channels (rather than four channels) is shown in Fig. 2. Class membership of a new normalized vector (event) is then determined by using Bayesian inference assuming each odorant has an equal prior probability. Details regarding pattern recognition techniques can be found in the following reference (Theodoridis and Koutroumbas 1999).

Quadro-probe Longevity and Odor Classification Longevity After conducting the plume-strand-mixing trials in experiments 2 and 3, each Quadro-probe preparation was maintained and tested at several time intervals, ending at 24 h after the initial wind tunnel tests. For the responses of the antennal array to strands from a plume of each of the odorants 1 and 2 and also to strands of the mixture of the two odorants on a single filter paper (odor 3), the subsequent analyses of odor strand classification were carried out and compared as scatter diagrams to those that were made by the system during the first hour of recording.

Maximum EAG Longevity To determine the maximum longevity of antennal responsiveness using another version of the whole-body preparation, a male *T. ni* moth was restrained on a Plasticine block with U-shaped thin copper wires. A fine-tip reference capillary glass electrode filled with 0.5-M KCl solution was inserted into a compound eye, and another capillary glass electrode filled with electro-conductive gel was brought into contact with antennal tip after cutting a few terminal antennal segments. The antenna was then placed in a charcoal-filtered humidified continu-

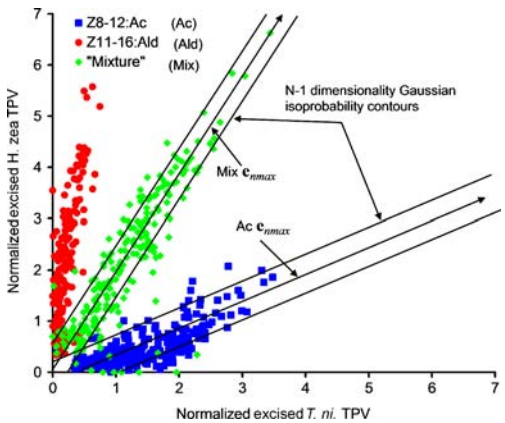

Fig. 2 Two-channel scatterplots of events obtained from four-channel simultaneous recordings of excised antennae from male *Trichoplusia ni* and male *Helicoverpa zea* antennae. These data were thresholded at 100 μV, as described in the text. Two-dimensional eigenvectors (purely illustrative) corresponding to the largest eigenvalues for Z8-12:Ac and mixture are shown. Isoprobability contours for these odors are drawn on either side of the eigenvectors

ous airflow (600 ml/min) at ~3 cm from the air outlet of a glass tube (8 mm ID). Stimuli were delivered by giving single 30-ms puffs (2-ml volume) of charcoal-filtered air through a large opening of a Pasteur pipette containing a filter paper strip (8×40 mm, Whatman no. 1) containing 0, 1, 10, or 100 ng of Z7-12:Ac, while the small opening of the Pasteur pipette was placed in the airflow through a small (3-mm diameter) opening in the glass airflow tube. EAG responses were measured at several different times each day until no EAG responses were observed. Three antennal preparations were tested; three consecutive stimuli were delivered at each measurement time.

Results

Experiment 1 In the wind tunnel, EAG responses to clean air (i.e., baseline activity) were usually lower than 50 μV at various airflow rates. Although some drift of baseline amplitude was observed in some antennae, it did not appear to significantly affect our chemosensor system performance. When two male *H. zea* antennae and two male *T. ni* antennae were used for the Quadro-probe and Z11-16:Ald, Z7-12:Ac, and 1:1 mixture of these two compounds were tested (all three odors emitted from single filter papers), *H. zea* antennae showed strong responses to Z11-16:Ald and the mixture (Fig. 3 Ald, mixture), and *T. ni* antennae showed strong responses to Z7-12:Ac and the mixture (Fig. 3 Ac, mixture).

The EAG depolarizations in response to odor strands from the two antennae of the same species were synchronized (Fig. 2) and exhibited consistent response proportionality over a wide range of pheromone strand flux strengths (Fig. 4a). The ratio of EAG amplitudes in response to the two-component mixture of *H. zea* antennae and *T. ni* antennae also was maintained regardless of the flux (amplitude of the EAGs) of the mixture odor strands (Fig. 4b). The Quadro-probe system thus was able to classify and discriminate the strands of these three different

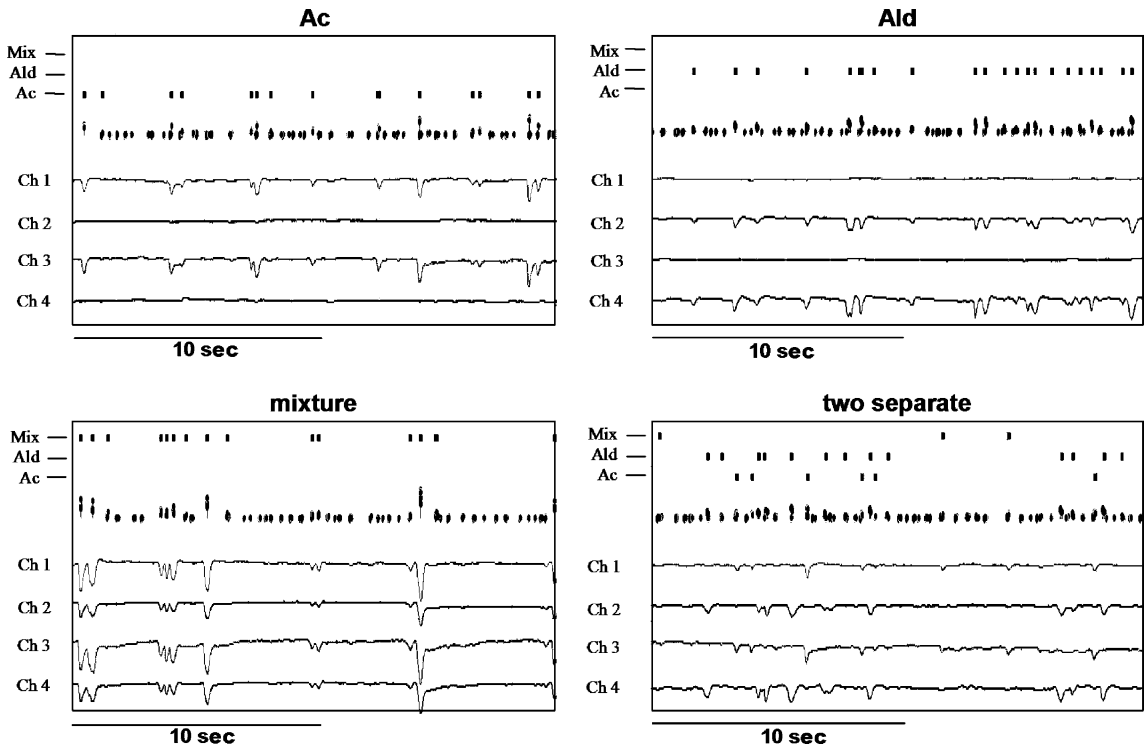

Fig. 3 Recordings from the four-antenna chemosensor system in different odor plumes in the wind tunnel. *Horizontal scale bar* 10 s. *Vertical scale* is 4 mV separation between channels. Each depolarizing peak (downward deflections in the four continuous-time traces) was an EAG response to contact with an individual odor filament. Four different moth antennae were used for the four-channel chemosensor probe. Ch 1 and ch 3 are male *T. ni*. Ch 2 and 4 are male *H. zea*. The probe was placed 1.5 m downwind from the filter paper test odor dispensers. Wind velocity was maintained at an average of 50 cm/s. The system was "trained" first to a plume of "Ac" (*Z*7-12:Ac), then to "Ald" (*Z*11-16:Ald), and finally to a mixture of the Ac and Ald placed on a single source ("mixture"). These sources were then used in the test situations illustrated here. The two separate sources of Ac and Ald were placed 5 cm crosswind from each other. *Lollipop-type symbols* along the *fourth line* from the *top* indicate depolarization "events" on individual channels that were detected by the system, and the *height* of each *oval* indicates the amplitude of each depolarization; channels are not indicated. Among these signals, those over an operator-determined threshold level were further analyzed and classified as Ac (*lowest row* of *squares*), Ald (*middle row* of *squares*), or a mixture of the two (*upper row* of *squares*)

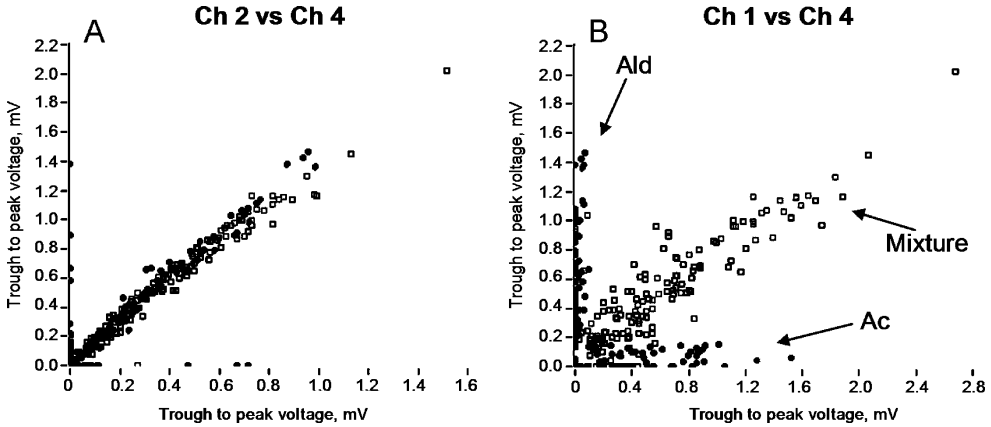

Fig. 4 The response amplitude relationships between two different antennae in the array, illustrating the profiles generated between two *T. ni* antennae (**A**, channel 2 vs. channel 4) and one *T. ni* and one *H. zea* antenna (**B**, channel 1 vs. channel 4) when exposed to strands from single filter paper sources of the Ac, Ald, or mixture of Ac + Ald, respectively. Regardless of EAG depolarization amplitude, same-species antennae (**A**) show a high-fidelity linear relationship regardless of the odor strand flux they are sampling. Two different species' antennae (**B**) also exhibit high-fidelity linear relationships regardless of the strand flux strength, but now these are exhibited in three distinct odor spaces that are characteristic of the three odors. Multidimensional *k*-nearest-neighbor analysis using clusters from all combinations of the four channels was the basic criterion used by the algorithm for odor classification

pheromone odors over a wide range of EAG amplitude flux with high accuracy (Table 1).

There was no significant difference in odorant recognition performance of the algorithm whether the training session was 20 or 60 s long (Table 1; there were no significant differences in odorant recognition performance when the tests were conducted at distances closer to the source than the 1.5 m training distance, except for Z11-16:Ald being less often correctly identified at 0.5 m than at the two longer distances). *P* values obtained from 2×2 (correct/incorrect × 20/60 s) chi-squared tests are listed in Table 1. Over all trials and all three test distances from the source, this experiment showed 98%, 86%, and 86% correct odor identification rates for Z11-16:Ald, Z7-12:Ac, and their mixture on a single source, respectively. When two different point odor sources (Z11-16:Ald and Z7-12:Ac) were placed 5 cm laterally apart at the usual upwind position in the wind tunnel, the Quadro-probe chemosensor system identified the individual strands from the confluent plumes of the two odors, plus instances in which the strands coincided within the 160 ms time window, registering a classification as the mixture (Table 2).

Experiment 2 The degree of synchronous arrival of plume strands arriving at the probe within the 160 ms time window conforms to the prediction of a higher frequency of occurrence of mixed strands when the sources are very close together. When the two pheromone odorant sources were separated laterally by 1 or 2 cm, there was a greater number of strands per second contacting the four-antenna probe downwind that were classified as "mixture" than when the sources were separated by 5 or 10 cm (Fig. 5). A mean of 1.75 strands per second was classified as being mixed at 1-cm filter paper separation compared to 0.69 strands per second at 5-cm separation and 0.34 strands per second at 10 cm separation that were classified as being mixed (Fig. 5).

The frequency of mixed strands (1.75 per second) as a proportion of the frequency of all three types of strands classified by the system (3.39 per second) was 0.52 at the 1-cm filter paper spacing, whereas it was 0.12 at the 10-cm spacing (0.34 per second mixed versus 2.93 per second total strands (Fig. 5); at the 5-cm spacing, this proportion was 0.18 (0.69 per second mixed versus 3.92 per second total strands). At the 2-cm filter paper spacing, there was

Table 2 Identification performance of the four-antenna chemosensor system for odor strands of two sex pheromone components, (*Z*)-7-dodecenyl acetate (Ac) and (*Z*)-11-hexadecenal (Ald), in a wind tunnel

Odor stimuli	Percent ratio identified as (mean±SE)			Total no. of EAG signals	Number
	Ac	Ald	Mixture		
Ac	86.1±6.41	5.4±1.30	8.5±5.96	66	3
Ald	2.4±2.38	97.6±2.38	0.0±0.00	75	3
Mixture of Ac and Ald on single source	2.6±1.29	11.4±5.56	86.0±5.18	149	5
Ac and Ald on two separate sources placed laterally 5 cm apart	38.7±7.75	42.3±6.22	19.0±4.21	509	13

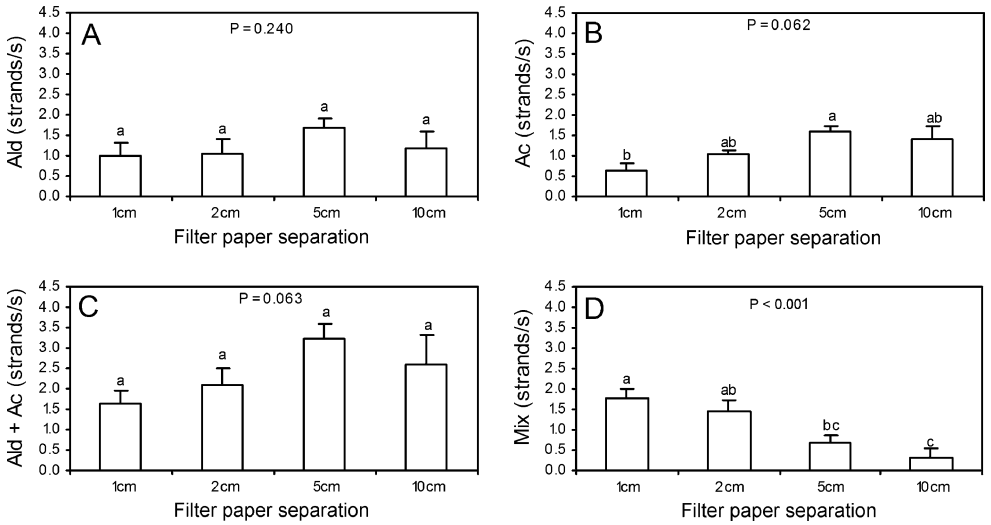

Fig. 5 Histograms (mean±SE) of the response frequencies, per second, of classified odor strands using a whole-body Quadro-probe preparation placed 1.5 m downwind of two closely spaced filter paper odor sources in the wind tunnel. Trials were conducted by using a filter paper emitting Z11-16:Ald and a second filter paper emitting Z7-12:Ac that were separated by 1, 2, 5, and 10 cm crosswind at the upwind end of the tunnel. EAG responses from the probe were classified as **a** Z11-16:Ald strands (Ald), **b** Z7-12:Ac strands (Ac), or else **d** "mixed" strands arriving coincidentally on the four-antenna array (mix). The sum of single strands classified as either Ald or Ac is shown in **c**. The more widely the filter papers were spaced, the less likely were plume strands to be judged as "mixed" (**d**) by the Quadro-probe system. A multidimensional Gaussian distribution was the basis for the classifier algorithm. N=3 antennal preparations, with two blocks each. Two-way (distance, block) ANOVA was performed on a general linear model of the strand frequencies of each odor, yielding P values of 0.240, 0.062, 0.063, and <0.001 for Ald, Ac, Ald + Ac, and mix, respectively. Tukey's pairwise comparisons resulted in 95% confidence groupings indicated by *lettering* located *above* each *bar*; means having no letters in common are significantly different at $P \leq 0.05$. The mean number of strands classified (all three odors) in each of the replicates in this experiment was 257 for the 1-cm filter paper separation, 276 for 2 cm, 262 for 5 cm, and 138 for 10-cm separation. Total strand frequency (all three odors) diminished as spacing increased, going from 3.84, 3.52, and 3.94 to 2.93 per second at 1-, 2-, 5-, and 10-cm filter paper separation, respectively

nearly as high a proportion (0.41) of mixed strands as at the 1-cm spacing (0.52). At 2 cm, the frequency was 1.44 per second mixed versus 3.52 per second total strands (Fig. 5). Thus, the asynchronous arrival of pheromone odor strands from two different pheromone sources can be detected and discriminated by the Quadro-probe system and varies according to odor source separation distance.

Experiment 3 Although the proof-of-concept ability of the insect-antenna-based probe plus software to discriminate odor strands separated in time downwind was demonstrated in experiment 2, we conducted a third study involving citronellal, a plant-volatile-based compound that provided a non-pheromonal background-noise plume against the signal from a pheromone plume. This situation that might occur when a female moth emits pheromone against a background of point source floral odors or induced plant volatiles from leaves.

Following training to citronellal, Z11-16:Ald, and a mixture of the two emitted from a single filter paper, the whole-moth-body 4-antenna system classified a greater frequency of odor strands as "mixture" when the plant- and pheromone-based sources were separated by 1 or 2 cm than when they were more widely separated by 5 or 10 cm (Fig. 6). A mean of 1.62 strands per second were classified as being mixed at the 1-cm filter paper separation compared to 0.56 and 0.28 strands per second that were classified as being mixed at the 5- and 10-cm separations, respectively.

The mixed strand frequency (1.62 per second) as a proportion of the total strand frequency of all three odors (3.45 per second) was 0.47 at the 1-cm filter paper spacing, whereas it was 0.10 at the 10-cm spacing (0.28 per second mixed strands versus 2.83 per second total strands (Fig. 6). At the 5-cm spacing, this proportion was 0.13 (0.56 per second mixed strands versus 4.24 per second total strands). At the 2-cm filter paper spacing, the proportion of mixed strands was 0.24 (0.87 per second mixed versus 3.56 per second total). Thus, the asynchronous arrival of plant odor strands and pheromone odor strands was detected and discriminated by the Quadro-probe system. As in experiment 2 that used two pheromone sources, the degree of synchronous arrival of plant odor strands and pheromone strands arriving at the probe within the 160-ms time window conforms to the prediction of a higher frequency of occurrence of mixed strands when the sources are very close together.

Whole-Body Quadro-probe Antennal Longevity The responsiveness of the Quadro-probe system array was preserved over 24 h (Fig. 7). The scatter diagrams show normalized training data (TPVs) obtained during three trials. Information obtained from two antennae are shown

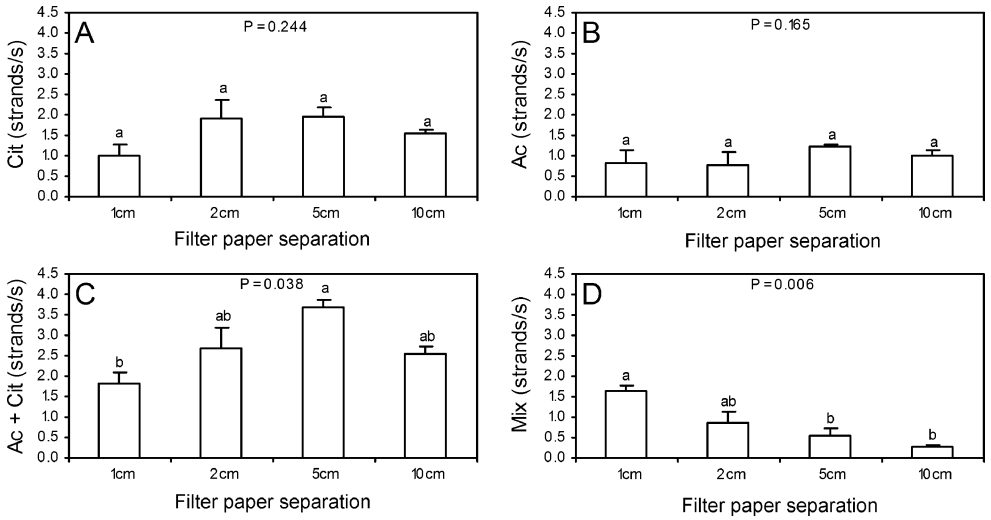

Fig. 6 Histograms (mean±SE) of the response frequencies, per second, of classified odor strands using a whole-body Quadro-probe preparation placed 1.5 m downwind of two closely spaced filter paper odor sources in the wind tunnel. Trials were conducted by using a filter paper emitting citronellal and a second filter paper emitting Z11-16:Ald that were separated by 1, 2, 5, and 10 cm crosswind at the upwind end of the tunnel. EAG responses from the probe were classified as **a** citronellal strands (Cit), **b** Z11-16:Ald strands (Ald), or else **d** "mixed" strands arriving coincidentally on the four-antenna array (mix). The sum of single strands classified as either Ald or Cit is shown in **c**. The more widely the filter papers were spaced, the less likely were plume strands to be judged as "mixed" (**d**) by the Quadro-probe system. A multidimensional Gaussian distribution was the basis for the classifier algorithm. N=2 antennal preparations, with two blocks each. Two-way (distance, block) ANOVA was performed on a general linear model of the strand frequencies of each odor, yielding P values of 0.244, 0.165, 0.038, and 0.006 for Cit, Ac, Cit + Ac, and mix, respectively. Tukey's pairwise comparisons resulted in 95% confidence groupings indicated by *lettering* located *above* each *bar*; means having no letters in common are significantly different at $P \leq 0.05$. The mean number of strands classified (all three odors) in each of the replicates in this experiment was 383 for the 1-cm filter paper separation, 418 for 2 cm, 459 for 5 cm, and 414 for 10-cm separation. Total strand frequency (all three odors) diminished as spacing increased, going from 3.45, 3.54, and 3.80 to 2.83 per second at 1-, 2-, 5-, and 10-cm filter paper separation, respectively

in each panel, after strand detection. Training data obtained at 0 and 24 h for trial 1 are shown in Fig. 7a, b, respectively. Similarly, training data collected at 0 and 24 h are shown in Fig. 7c, d for trial 2 and Fig. 7e, f for trial 3. Only two channels were recorded in trial 1, while four (two of each species) were recorded (only two shown) in trials 2 and 3. The reason trial 1 has less data near the origin is that there is less noise immunity with only two channels. To show that the live preparations are effective for at least 24 h, we measured the performance of the classification system in terms of error rate for each trial at 0 and 24 h. We used the empirical error rate measure on re-substituted training data with the two-stage classification method outlined in Myrick et al. (2008). Briefly, this method uses a detection procedure (stage 1) to separate "significant" (compared to baseline activity) strands from "insignificant" strands prior to classification using a k-NN method (stage 2). Stage 2 allows for the rejection of ambiguous responses, which was not utilized here (ambiguity reject parameter of 0.0). The percent correct classification for each odor is shown in each panel with the order being (clockwise from the upper left) Z7-12:Ac, mixture, and Z11-16:Ald (Fig. 7).

Single Antenna Longevity When a different form of the whole-body preparation was employed and monitored by using single *T. ni* antennae, EAG responsiveness to different amounts of the major sex pheromone component, Z7-12:Ac, lasted for longer than 60 h (Fig. 8). The EAG responsiveness of the whole-body preparation was maintained without reduced amplitude compared to the first hour for greater than 50 h, with a relatively steep drop-off thereafter.

Discussion

Whole antennae have been used for EAGs that report the peak-to-trough fluctuations in odor strand flux that occur on a several Hertz basis in natural odor plumes (Vickers and Baker 1994; Baker et al. 1998; Vickers et al. 2001; Bau et al. 2002, 2005; Vickers 2006). Experimentally pulsed odorant strands showed that there is a temporal resolution of EAGs that is much higher than this: up to 25 Hz in *Bombyx mori*, 5 Hz for *Lymantria dispar* (Bau et al. 2005), 33 Hz for *Spodoptera exigua* and *Cadra cautella*, and up to 25 Hz in *Pectinophora gossypiella* (Bau et al. 2002). Characterization of the antenna as an information transducer revealed a simple linear first-order low-pass filter with a −3-dB bandwidth of approximately 10 Hz (*S. exigua*) and information carrying capacity of up to 37 bits/s. (Justice et al. 2005)

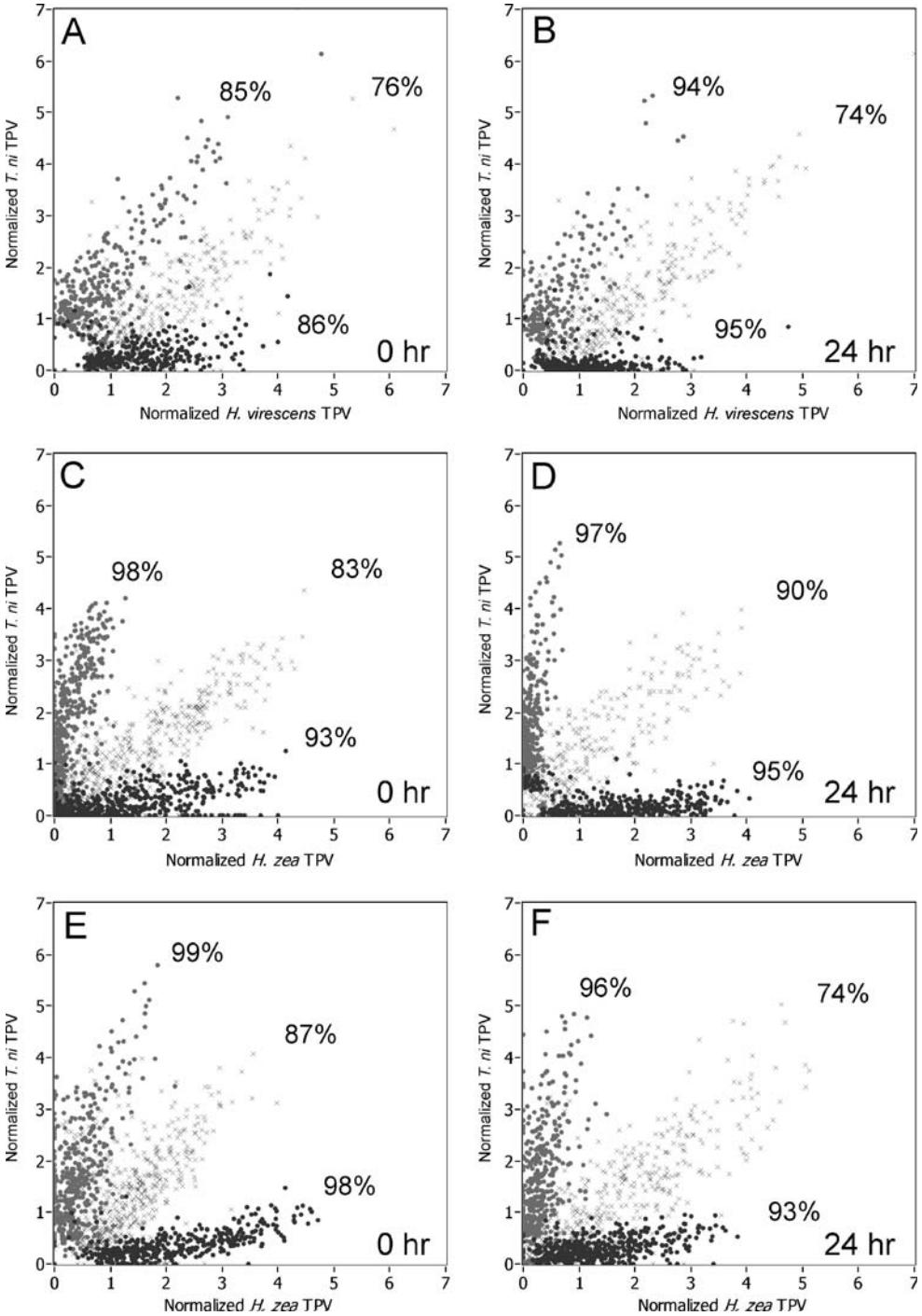

Fig. 7 Training data and classification accuracies (in percent) obtained from the Quadro-probe whole-body preparations over 24 h in response to strands of Z11-16:Ald, Z7-12:Ac, and the mixture of the two on the same filter papers presented as single-point-source odor plumes in the wind tunnel. *TPV* is trough-to-peak voltage. The four-antenna preparation was placed 1.5 m downwind of the filter paper source. Each *shaded symbol* is a classified response to an individual odor strand. *Black* = "aldehyde," *dark gray* = "acetate," *X symbol* = mixture. Highest depolarization amplitudes in response to odor strands were approximately 2 mV. **a**, **b** Trial 1 training data at 0 and 24 h, respectively. **c**, **d** Trial 2 training data at 0 and 24 h, respectively. **e**, **f** Trial 3 training data at 0 and 24 h, respectively. Each trial is a different whole-moth preparation. Error rates obtained (see text) for each re-substituted set of training data are converted into percent correct and labeled near the data corresponding to each odor; the percentages correspond to Ac, mix, and Ald in *clockwise* direction on each panel. Only one *T. ni* and one *H. zea*/*virescens* whole-moth EAG channel could be used for each two-dimensional depiction. N=3 replicates (trials)

Despite these previous successes of using single-antenna EAGs, quantification of the degree of odor strand mixing within overlapping odor plumes has not been possible to date because of an inability not only to detect but to classify on a strand-by-strand basis the odor strands in plumes comprised of different odorants. The present study shows that we can resolve incompletely mixed intertwined strands of odor within confluent plumes and classify their composition.

Our results show that two confluent plumes that on a time-averaged basis would appear to be perfectly mixed are in fact incompletely mixed when examined with the resolution provided by the insect antennal flux-detector tissue. We should now be better able to quantitatively relate an insect's behavior in response to complex odor plume interactions to the degree of mixing of plume strands that the insect experiences downwind. Experiments can be performed now not only with experimental pulses but also

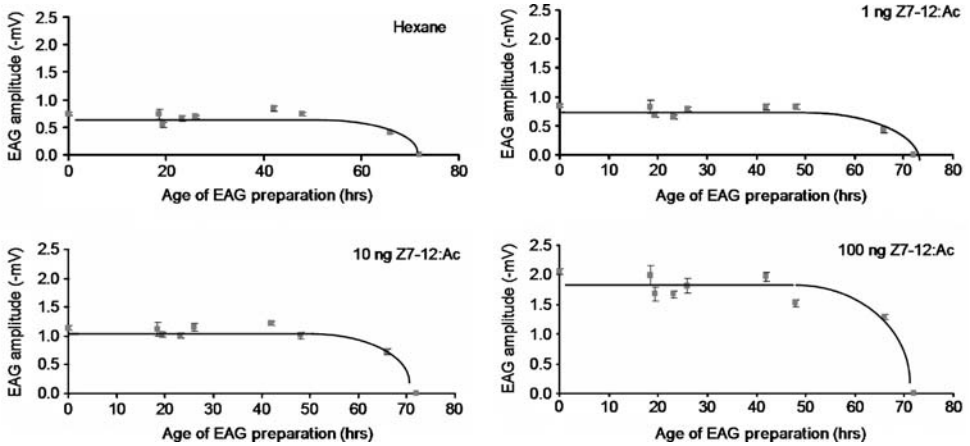

Fig. 8 EAG responsiveness of a whole-body preparation of male *T. ni* to different doses of Z7-12:Ac acetate over time (Mean± SE, $N \approx 7–45$)

with more natural point source plumes that overlap to different degrees.

The ability of the Quadro-probe to quantify incompletely mixed strands of odors that emanate from two or more different point sources is an apparent advantage of the insect antenna-based chemosensor over many other existing biomimetic artificial nose systems. In our experiments, only a small percentage of strands from two confluent plumes separated by more than 5 cm at their source in the wind tunnel arrived closely enough in time that the system declared them to be a "mixture." Further improvements on the system's range of odor measurement abilities will, of course, improve its applicability to more varied semi-ochemical systems that involve different combinations of odor plumes. In addition, improvement of the temporal resolution of the analysis software by further reducing its current 160-ms time window for declaring strands to be mixed vs. separate will more closely mirror insect olfactory systems' temporal resolution abilities.

Insects have been shown in behavioral experiments to discriminate between two confluent odor plumes as opposed to a blend of the two odorants placed on a single source (Witzgall and Priesner 1991; Liu and Haynes 1992; Baker et al. 1998). In behavioral studies, *H. zea* was able to distinguish between experimentally generated odor strands separated by 0.001 s in time and 1 mm in space (Baker et al. 1998; Fadamiro et al. 1999). There is also evidence in scarab beetles that puffs of two different pheromone components that arrive on ORNs with less than 1-ms separation cause different levels of ORN stimulation than when they arrive simultaneously (Nikonov and Leal 2002). Thus, the resolving power for this olfactory feat, at least in part, appears to result from mixture interactions that occur "on-site" (Baker et al. 1998; Todd and Baker 1999) between intimately associated ORNs that are co-compartmentalized within individual sensory hairs, pegs, or plates.

One of the most fragile aspects of any insect-antenna-based chemosensor has been its longevity of responsiveness. This has typically been less than 1 h (van Giessen et al. 1994; Hardie et al. 1994; Visser et al. 1996; Visser and Piron 1997; Park et al. 2002). The limited lifetime of the preparation has been a limitation in various other studies that employ living tissues as olfactory chemosensors (Kuwana and Shimoyama 1998; Kuwana et al. 1999; Schroth et al. 1999; Schütz et al. 1999). However, we have shown in this study that the longevity of the Quadro-probe EAG preparations can be improved significantly by using the entire moth bodies (Park and Hardie 1998; Park et al. 2002). Indeed, when whole-body preparations were used instead of isolated antennae, EAG responsiveness was shown to last up to 50 h with no decline in amplitude, and the fidelity of classifying odorants and blends of odorants by the multiantenna system could last as long as at least 24 h without significant reduction of response.

The extremely high sensitivity of insects' olfactory systems has been well documented (Angioy et al. 2003). A recent comparison between the peripheral position of this insect system, the antennae, and one of the most sensitive commercial electronic noses, the Cyranose 320, showed that insects have one to two orders of magnitude better sensitivity than the electronic nose (Rains et al. 2004). A BioFET chemosensor was developed by connecting an insect antenna to a field-effect transistor, showing that it could detect 1-ppb concentration of *Z*-3-hexen-1-ol (Schöning et al. 2000). We can hope that fully non-tissue-based chemosensors (Schaller et al. 1998; Walt et al. 1998; Drake et al. 2003) can be developed that mimic some of the features of insect antennae, especially their high sensitivity flux-detection abilities that can be of use for detecting and classifying strands of different odors in overlapping plumes.

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