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Improvement of signal-to-noise ratio in electroantennogram responses using multiple insect antennae

Kye Chung Park *, Thomas C. Baker

Department of Entomology, 407 Science II Building, Iowa State University, Ames, Iowa 50011, USA

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Abstract

Using an array of insect antennae connected in series or in parallel, electroantennogram (EAG) responses and noise levels were investigated in an attempt to improve signal-to-noise (S/N) ratio and sensitivity. Both the EAG response amplitude and noise level increased when the antennae of male *Helicoverpa zea* (Lepidoptera: Noctuidae) were connected in series. Due to lower relative increase in noise level than EAG amplitude as the number of antennae increased, the S/N ratio was also significantly improved by the serial connection. As a result the sensitivity of EAG was improved by the serial connection, which showed ca. ten-fold improvement in the threshold detection levels compared with a single antenna when four antennae were connected in series. In contrast to the serial connection, there were no differences in EAG amplitudes and overall noise levels when different numbers of antennae were connected in parallel. When only large-amplitude noise was taken into account, however, the S/N ratio was somewhat improved by the parallel connection. The frequency of overall noise remained at the same level both in the serial and in the parallel connection. However, the frequency of the large-amplitude noise increased in serial connection but decreased in parallel connection. The present study clearly indicates that both the sensitivity and S/N ratio of the EAG biosensor could be significantly improved by using the multiple antennal connections.

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Keywords: Antennae; Biosensor; EAG; Electroantennogram; *Helicoverpa zea*; Signal-to-noise ratio; S/N ratio

1. Introduction

The electroantennogram (EAG) technique has been widely used to identify pheromones and kairomones in a number of insect species (c.f., Arn et al., 1975; Cork, 1994; Cossé et al., 1995) ever since the technique was first developed more than 40 years ago (Schneider, 1957). The legacy of the EAG thus far has been as a biosensor that responds somewhat selectively, depending on the species used, to certain classes and in some cases, isomers, of various volatile compounds (Roelofs and Comeau, 1971; Leal, 1999; Park et al., 2002). EAG sensitivity has not been an issue for this application in natural products chemistry, because it has surpassed that of the capillary gas chromatograph and mass spectrometer

(Zhang et al., 1997; Leal et al., 1996; Leal, 1999). During the last couple of decades researchers have been exploring a new application for the EAG technique in analyzing odor plume structure and monitoring odor filament frequency and concentration in real time, even under field conditions, and relating these to behavior (Baker and Haynes, 1989; Sauer et al., 1992; Karg and Sauer, 1995; Milli et al., 1997; Pers van der and Minks, 1998). More recently, several attempts have been made to expand this application into using insect antennae as biosensors for detecting selected volatile compounds in the ambient environment, which have been encouraging (Schütz et al., 1997; Kuwana and Shimoyama, 1998; Schöning et al., 1998; Kuwana et al., 1999; Schütz et al., 1999; Park et al., 2002).

In order to maximize the usefulness of the EAG as an olfactory biosensor, it is important to achieve an optimal signal-to-noise (S/N) ratio while increasing overall sensitivity. As EAG signals are increasingly amplified and variously processed by electrical equipment, various

* Corresponding author. Tel.: +1 515 294 7264; fax: +1 515 294 5957.

E-mail address: kcpark@iastate.edu (K.C. Park).

extrinsic noise can be superimposed onto the EAG signals as well as intrinsic noise such as the activity of insect cells. Extrinsic noise such as 50 or 60 Hz main power noise and surrounding electromagnetic noise can of course be reduced by using a proper electrical circuit filtering system, plus appropriate shielding and grounding. In theory, the S/N ratio of EAG signals with randomly generated noise can be improved by a proper connection using multiple inputs, which have successfully been used in signal processing and information technology. Indeed, a few studies have shown that EAG amplitude could increase by using multiple antennae connected in series (Moore, 1981). However, it has so far been unclear whether the sensitivity and the S/N ratio was actually improved by the serial connections because the changes in noise level were not described. The present study was carried out, therefore, to investigate further the effect of using multiple antennae connected in series or in parallel on EAG amplitude and noise by measuring both amplitudes of response and noise levels and comparing them to single antennal responses.

2. Materials and methods

2.1. Insects

A colony of the corn earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae), was maintained in a rearing room in Iowa State University under the constant temperature (20 ± 1 °C) and light (14L : 10D) condition. Pupae were sexed and kept in separate cages until adult emergence. Adult moths were kept in larger cages and provided with water and 10% sugar solution. Two- to three-day-old male moths were used for the experiments.

2.2. EAG recording

Antennae of male *H. zea* were isolated from the head with micro-scissors. The excised antennae were mounted across the tip of glass capillary tubes (1 mm OD, 15 mm long, Drummond Scientific Co., Broomall, USA) fixed on a plasticine block (see Fig. 1 for the antennal connections). A small amount of electroconductive gel was applied on the tip of the glass capillary tubes prior to the antennal mounting. For EAG recordings from the antennae connected in series, four antennae were mounted in zigzag arrangement on the glass capillary tubes (Fig. 1 top), and the basal part of the first antenna (Fig. 1 position A) was connected to a glass electrode (serving as a reference electrode) (0.86 mm ID, 3 cm long, A-M Systems Inc., Everett, USA.) that was filled with electroconductive gel (Spectra®, Parker Laboratories Inc., Orange, USA). A few distal segments of the first antenna were cut off and brought to contact via gel with the base of the second antennae (Fig. 1 position B).

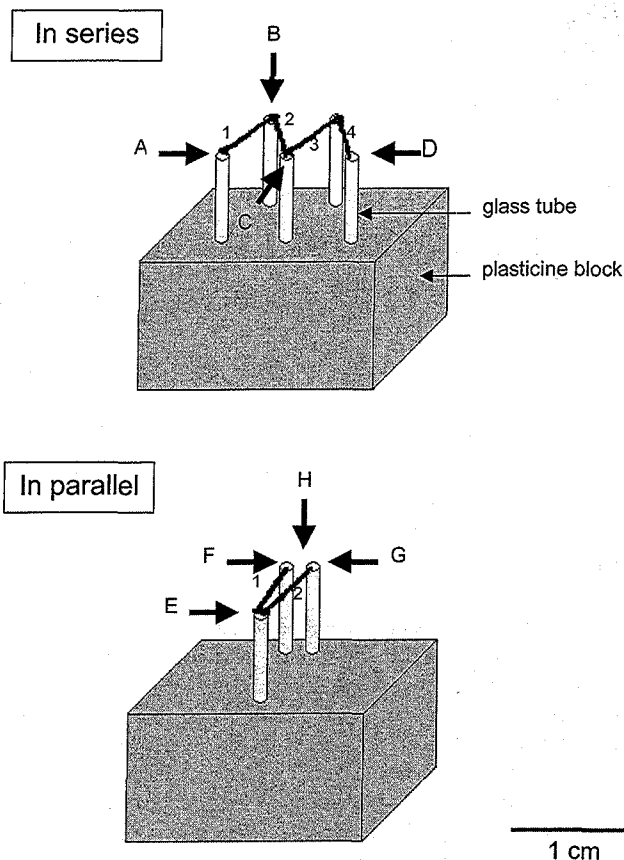


Fig. 1. Antennal preparations and the connections in series and in parallel. Antennae were mounted on the top of small glass tubes placed on a plasticine block. The numbers indicate the antennae, and the letters indicate the position of the electrodes (see text for details).

The second antennal tip was then cut and placed onto a third gel-tipped capillary and so on for all four antennae. Another gel-filled glass electrode was used as a recording electrode and the position of electrodes was controlled with micromanipulators. Electrical contact between the antennae was maintained by the application of the electroconductive gel. The four antennae were closely positioned to keep the distance between two antennae less than 5 mm to facilitate their exposure to the same odor puff. The antennae were positioned in the middle of charcoal-filtered and humidified main air stream (1 L/min) for the delivery of stimulus. Ag-AgCl wire was used to maintain electrical continuity between the electrodes and a high input impedance headstage pre-amplifier. The EAG signals through the preamplifier were further amplified and processed with a PC-based signal processing system (Syntech®, The Netherlands). A series of experiments was commenced by recording EAG responses from antenna #1 by connecting the recording electrode to the tip of the antenna #1 (position B). Then, the recording electrode was moved and connected to the tip of antenna #2 (position C), making a closed circuit with both antennae #1 and #2 in series. Thus, EAG responses were now recorded from two

antennae connected in series. Finally, the recording electrode was moved and connected to the tip of antenna #4 (position D), resulting in four antennae being recorded from in series in a closed circuit. The series of recordings was repeated three times for each preparation.

For EAG recordings with antennae connected in parallel two isolated antennae were mounted on three small glass capillary tubes on a plasticine block, using electroconductive gel (Fig. 1 bottom). The bases of both antennae contacted electrically each other via electroconductive gel, and were connected to a glass electrode serving as a reference electrode (position E). The cut distal tip of each antenna was then positioned on a separate glass pole, but close to each other (< 1 mm between the tips). A series of in-parallel EAG recordings was commenced by placing a recording electrode in contact with the tip of antenna #1 (position F). Then, the recording electrode was connected to antenna #2 (position G). Finally, the recording electrode was placed in-between the tips of two antennae to make electrical contact with both antennal tips (position H). The complete sequence of in-parallel recordings was repeated three times.

2.3. Chemical stimulus and odor application

Serial dilutions of a major female sex pheromone component, (Z)-11-hexadecenal (Z11-16:Ald) (chemical and isomeric purity $> 99.5\%$, Aldrich®, USA) were prepared in hexane to make 0.0001–10 $\mu\text{g}/\mu\text{l}$ solutions. A ten-microliter aliquot of each test solution was applied onto a piece (6×32 mm) of filter paper (Whatman® No 1, USA), and the filter paper strip was inserted into a glass Pasteur pipette (146 mm long, Fisher Scientific®, USA). The tip of the pipette was inserted into a small hole (3 mm diameter, 12 cm from the outlet) of a main airflow tube (12 mm diameter, 17 cm long) where a continuous, charcoal-filtered and humidified airflow (1 L/min) was blown through onto the antennal preparation. A 0.5-s puff of charcoal-filtered airflow (10 ml/s) was injected through the large end of the Pasteur pipette odor cartridge for stimulation, using an electronically controlled airflow controller (SFC-2, Syntech®, The Netherlands).

3. Results

Although antennae were isolated from the head, their EAG responsiveness lasted more than one hour without significant decline (data not shown). Waveforms of the EAG responses with multiple antennae were similar to those of single antenna (Fig. 2), showing rapid depolarization (average about 0.2 s of rising time) after stimulation and relatively slow recovery time (average about 3.0 s) (Figs. 2 and 3). Various noise was also observed

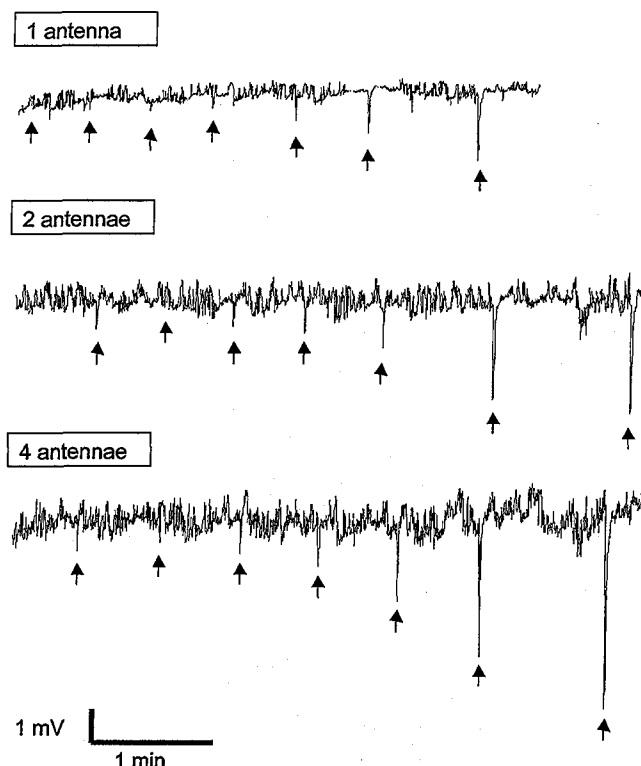


Fig. 2. Examples of EAG responses recorded with 1, 2 and 4 antennae connected in series in male *Helicoverpa zea*. The EAG recordings were successively made from same antennal preparation. Seven different doses of Z11-16:Ald, ranging 0–100 μg , were used for stimulations in each recording. Arrows left indicate EAG responses to 0, 0.001, 0.01, 0.1, 1, 10, and 100 μg of Z11-16:Ald, from left respectively.

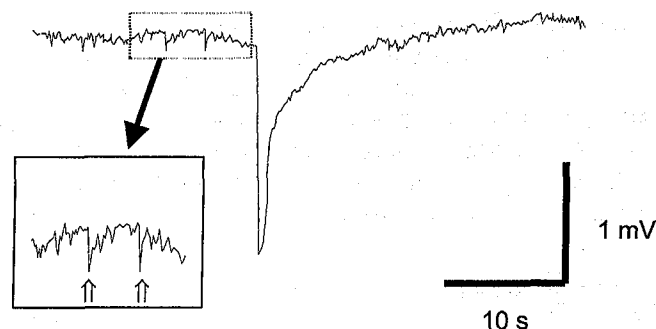


Fig. 3. An EAG response waveform of male *Helicoverpa zea* to 100 μg of Z11-16:Ald, showing a depolarizing EAG response and noise. Several relatively large noises (dotted ones in the box) and a number of small noises (not marked) are seen.

while no chemical stimulus was applied, and the noise could be classified, using the amplitude and frequency of the noise, into two classes: large, lower frequency noise (Fig. 3 marked with open arrows in the box); and small, higher frequency noise (Fig. 3). Regardless of the type of antennal connection, i.e. in series or in parallel, typical dose-dependent response characteristics were observed in the EAG responses of male *H. zea* to Z11-

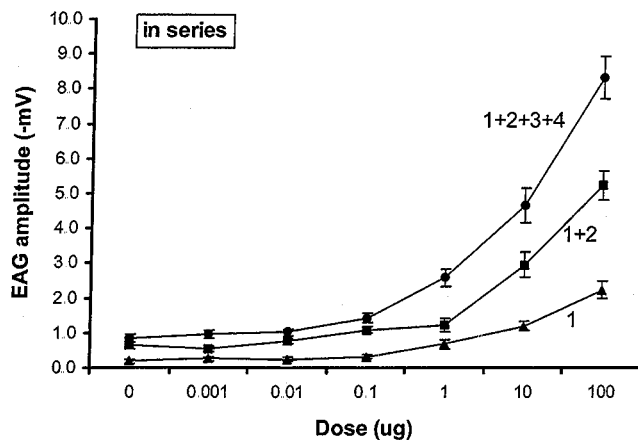


Fig. 4. EAG dose-responses of male *Helicoverpa zea* to various doses of Z11-16:Ald with different number of antennae connected in series (mean \pm S.E., $n = 11$). The EAG responses were measured from one antenna (1), two antennae connected in series (1 + 2), or four antennae connected in series (1 + 2 + 3 + 4).

16:Ald (Figs. 4 and 5). When no input connection was made the recording system showed $< 10 \mu\text{V}$ of intrinsic noise level with approximately 3.5 Hz of relatively constant frequency. The blank noise level remained at the same level when two electrodes were brought into a contact through a gel-filled glass capillary.

When the antennae were connected in series, the amplitude of EAG responses increased with increasing number of antennae (Fig. 4). Threshold dose eliciting significant EAG responses was also improved by the serial connection from $1 \mu\text{g}$ in one antenna to $0.1 \mu\text{g}$ in four antennae ($p < 0.05$, LSD test). Together with the EAG amplitude, the noise level of both small and large noise also increased with the increasing number of antennae in series (Table 1). In contrast, only large noise showed an increase in frequency by the serial connection, with no such changes being observed in the small noise. As a result, there was a significant improvement in the signal-to-noise (S/N) ratio (ca. a 50% increase

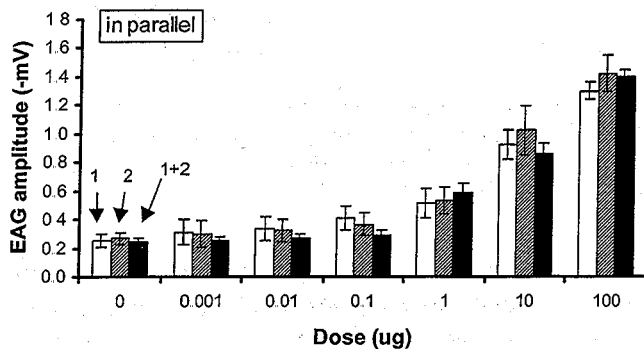


Fig. 5. EAG dose-responses of male *Helicoverpa zea* to various doses of Z11-16:Ald with different number of antennae connected in parallel (mean \pm S.E., $n = 9$). The EAG responses were measured from each of two different antennae (1 or 2), or two antennae connected in parallel (1 + 2).

with four antennae compared with a single antenna) by the serial connection.

When the antennae were connected in parallel, the amplitudes of EAG response remained unchanged regardless of the number of antennae (Fig. 5). The small noise showed no changes either in amplitude or frequency, but there were significant decreases in both amplitude and frequency of the large noise in the parallel connection (Table 2). The frequency of the large noise was reduced by approximately 50% when two antennae were connected in parallel. As a result, the S/N ratio for the large noise was improved by the parallel connection, although the S/N ratio remained unchanged when the overall noise was considered.

4. Discussion

When the antennae of male *H. zea* were connected in series or in parallel, the waveform of the EAG responses did not show any differences from that of single antenna, which was similar to a previous report showing an unchanged EAG response waveform in serial connection (Moore, 1981). This indicates that the antennae were placed close enough to respond to the stimulus pulse almost at the same time, without any significant delay in response time between the antennae. This also implies that each antenna kept similar time-response characteristics, especially in the rise time.

In EAG recordings, noise can be introduced into the system from various external sources such as electromagnetic fields, static electricity and 50 or 60 Hz main power, as well as from internal sources such as the activity of the cells and perturbations of the antennae. Higher frequency noise such as the 50 or 60 Hz mains noise can be almost completely removed by using a band-pass filter as used in our recording system. Such filtering has been very useful in slow potential DC recordings in particular. The small noise observed in the present study might have originated from external sources, because the frequency of their occurrence was not affected by various antennal connections and the amplitude was not reduced by the parallel connection. Much smaller ($< 10 \mu\text{V}$) intrinsic blank noise level of our recording system also indicates the origin of the small noises from external sources. In contrast, the large noise was likely coming from the antennae themselves, because both the amplitude and frequency of the large noise was significantly affected by both serial and parallel connections.

The increase of EAG amplitude in the serial connection observed in the present study has been reported in another study that showed approximately 3–4-fold increase in the EAG amplitude by connecting four antennae of *Spodoptera littoralis* in series (Moore, 1981). Although the major principle responsible for the

Table 1
EAG signal-to-noise (S/N) ratio with multiple antennae in series in male *Helicoverpa zea* (100 µg Z11–16:Ald)

Noise	Antennae	EAG signal (-mV)	Noise (-mV)	Noise frequency ¹²	S/N ratio ²
Overall noise ³	1	2.23±0.25	0.120±0.008	10.7	18.583 ^b
	1+2	5.24±0.42	0.229±0.019	11.5	22.882 ^{ab}
	1+2+3+4	8.31±0.61	0.304±0.021	12.1	27.336 ^a
Large noise	1	2.23±0.25	0.279±0.008	2.4 ^c	7.993 ^b
	1+2	5.24±0.42	0.556±0.016	4.4 ^b	9.424 ^{ab}
	1+2+3+4	8.31±0.61	0.755±0.023	6.1 ^a	11.007 ^a

¹ Noise frequency was expressed as number of noises/10 s

² Different letters indicate significant differences within a column in each type of noise (Duncan's Multiple Range Test, $p < 0.05$)

³ Overall noise includes large noise and small noise

Table 2
EAG signal-to-noise (S/N) ratio with multiple antennae in parallel in male *Helicoverpa zea* (100 µg Z11–16:Ald)

Noise	Antennae	EAG signal (-mV)	Noise (-mV)	Noise frequency ¹²	S/N ratio ²
Overall noise ³	1	1.30±0.06	0.089±0.008	13.1	14.607
	2	1.42±0.13	0.094±0.007	12.5	15.106
	1+2	1.40±0.05	0.088±0.007	13.1	15.909
Large noise	1	1.30±0.06	0.206±0.008	0.9 ^b	6.311 ^b
	2	1.42±0.13	0.252±0.008	1.3 ^a	5.635 ^b
	1+2	1.40±0.05	0.180±0.007	0.5 ^c	7.778 ^a

¹ Noise frequency was expressed as number of noises/10 s

² Different letters indicate significant differences within a column in each type of noise (Duncan's Multiple Range Test, $p < 0.05$)

³ Overall noise includes large noise and small noise

increase of EAG amplitude in the serial connections would be the same as in simple electrical circuits, overall EAG amplitude of multiple antennae in series was not exactly the same as the arithmetic summation of amplitudes of individual antennae. Regardless, it might be more important to improve EAG sensitivity by improving the S/N ratio rather than increasing signal amplitude. This means that the noise level should be taken into account as well as the EAG signal amplitude when sensitivity is concerned.

The present study first indicates that the S/N ratio could be significantly improved with the serial connection, which showed an improvement of EAG sensitivity by approximately ten-fold in the threshold dose with the serial connection using four antennae. The increase of noise frequency in the serial connections occurred mainly in the large noise, indicating that the large noise was being generated in a random manner. The improvement of the S/N ratio by serial connection, which was mainly due to a relatively lower increase of the large noise when compared to the EAG signal, may be explained by the following assumptions: 1) Large noise is intrinsic and random. 2) The large noise is generated mainly at different times in different antennae connected in series. Therefore, the amplitude of the large noise did not increase as much as the summated serial EAG response to stimulus did.

EAG responses in the parallel connection showed a different trend from our initial assumption. If the connection had similar characteristics to those of electrical circuits the amplitude of both EAG and noise should have remained at the same level in the parallel connection. Indeed, no differences in EAG amplitude in the parallel connection would be the same as would be expected in an electrical circuit. However, the decrease of amplitude and frequency of large noise in the parallel connection cannot be explained in this way, and this would also indicate the intrinsic origin and random generation of the large noise. Although the effect of parallel connection was not as great as in the serial connections, our results indicate that the S/N ratio of EAG responses can also be improved by parallel connection when only large noise is considered. In practice, the large noise would be more important than small noise because the large noise would be a main cause of false signals when the EAG system is used as an olfactory biosensor.

Even considering both small and large noise, however, the possibility for improvement of detection capability due to enhanced amplitude of the signal alone needs to be reconsidered here. Because the biosensor is mainly a flux detector that detects changes in odor concentration, even broad-band noise, if monitored for background levels in an equilibrated pre-stimulus state can be compared with a post-stimulus state by using peak-to-peak

changes. If the noise in a serially connected array is not due to a synchronous activity of all the units, and even if it is it has an identifiable temporal occurrence, then the imposition of a signal onto this background level that has a known (and different) temporal structure should allow the array's response to accurately report the signal as a discernable change in the biosensor's output. Alternatively, if the signal can reliably sum with the noise, not just be interposed between it, then detection of signal peaks can be good.

Our study mainly focuses on the EAG sensitivity and S/N ratio. Only single puffs of known stimulation timing and strength were used, which made EAG signal discrimination relatively easy. In practice, however, the frequency, strength, and timing of stimulus signals are often unknown. Depending on the frequency, regularity, and amplitude of signals and noise, several different hypothetical situations can be considered in this case (Fig. 6): A. S/N ratio = 1, but N is always occurring and due to different modality than odor S can sum with N, always (detection good). B. Again S/N = 1, but N occurs sporadically and infrequently. S rarely sums with N (no detection). C, D. S/N = 1, N occurs sporadically and infrequently but S time-course is frequent relative to N (detection good). E. S/N = 1, S has distinct waveform (detection good). Therefore, it is not just S/N ratio that can be important, but the ability of the system to detect change, either in temporal aspects, amplitude (due to S and N summation) or both. Thus, when combined with frequency, regularity, and waveforms of the signals and noise, the ability to discriminate signals from noise can significantly increase.

Recently, several attempts have been made to use insect antennae as an olfactory biosensor detecting low levels of volatile compounds in the ambient environment (Kuwana and Shimoyama, 1998; Kuwana et al., 1999; Schroth et al., 1999; Park et al., 2002). The main advantage of using insect antennae as olfactory biosensors comes from their high sensitivity and selectivity (Schütz et al., 1999). Their sensitivity comes from their fast response time, and therefore their ability to respond to the peak odor filament concentrations of 2–4 hz that occur in natural odor plumes (Baker and Haynes, 1989; Park et al., 2002). Although other types of artificial olfactory sensors and sniffing devices have been developed, most of them have exhibited relatively poor sensitivity, typically lower than a few ppm level, at least in part due to their slow response time and hence their ability to only detect the mean concentration (Walt et al., 1998; Kasai et al., 1999). It would thus be difficult to monitor volatile compounds present in the environment in real time with such devices especially when the volatile compounds of interest are present at extremely low concentrations. Various electronic noses have offered the advantage of increasing levels of discrimination ability over the years with arrays of differentially

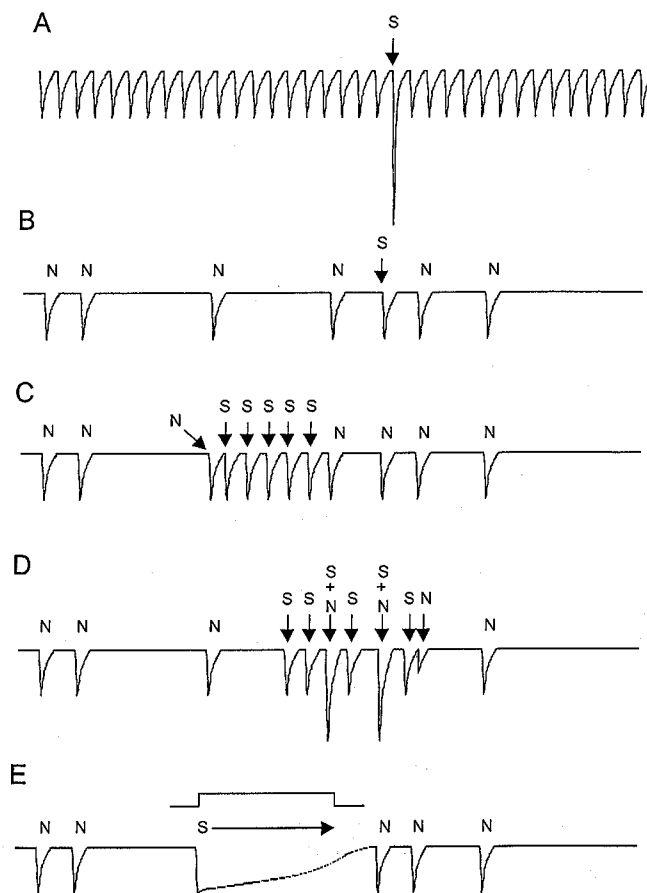


Fig. 6. Hypothetical EAG response discrimination amongst various possible noise in various situations (S: EAG signal, N: noise). A. Regular noise and a single EAG signal with larger amplitude. The EAG signal can easily be identified. B. Random noise and a single EAG signal. The EAG signal cannot be distinguished unless its amplitude differs from the noise. C. Random noise and regularly generated higher frequency EAG signals. The EAG signals in group could be identified by comparing the response frequency even though the amplitude is not much different between the EAG and noise. D. Random noise and regularly generated higher frequency EAG signals. Individual EAG signals could even be distinguished from noise if the EAG amplitudes are similar each other. E. Random noise and a single EAG signal with different waveform. The EAG signal can easily be distinguished by the difference in the waveform.

tuned inorganic sensor materials. A recent study has now shown that an array of four slightly differentially responsive insect antennae can be used to discriminate odor quality while taking advantage of EAG sensitivity (Park et al., 2002), which is crucial if the EAG technique is to be used for monitoring target volatiles amidst a background of other volatiles. Improvements in the sensitivity of the EAG, as shown here, could have significant impact on the development of sensitive and selective olfactory biosensors using insect antennae. The present study clearly suggests that detection sensitivity of EAGs can be significantly improved by using multiple antennal connections.

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