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Olfactory Sensory Neurons of the Asian Longhorned Beetle, *Anoplophora glabripennis*, Specifically Responsive to its two Aggregation-Sex Pheromone Components

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Abstract

We performed single-sensillum recordings from male and female antennae of the Asian longhorned beetle, *Anoplophora glabripennis*, that included as stimuli the two components of this species' aggregation-sex pheromone in addition to various general odorants. We compared the aggregation-sex-pheromone-component responses of olfactory sensory neurons (OSNs) to those of OSNs that responded to a variety of plant-related odorants. In the smooth-tipped, tapered, trichoid sensilla on the most distal antennal flagellomeres nos. 10 or 11 of both males and females, we found OSNs with high-amplitude action potentials that were tuned to the aldehyde and alcohol pheromone components and that did not respond to various plant-related volatiles. Because this OSN type responded to both the alcohol and aldehyde components it cannot be considered to be specifically tuned to either component. These large-spiking OSNs were co-compartmentalized in these sensilla with a second, smaller-spiking OSN responding to plant-related volatiles such as geraniol, citronellal, limonene, 1-octanol, nerol and citral. The large-spiking OSNs thus appear to be a type that will be involved in aggregation-sex pheromone pathways targeting a specific glomerulus in the antennal lobe and in generating pheromone-related behavioral responses in *A. glabripennis*. In other sensilla located in these distal antennal flagellomeres as well as those located more proximally, i.e., mid-length along the antenna on flagellomere nos. 4–7, we found OSNs in blunt-tipped basiconic sensilla that were responsive to other plant-related volatiles, especially the terpenoids, (*E,E*)-alpha farnesene, (*E*)-β-farnesene, β-caryophyllene, and eugenol. Some of these terpenoids have been implicated in improving attraction to pheromone-baited traps. Some of these same OSNs responded additionally to either of the two sex pheromone components, but because these OSNs also responded to some of the above plant volatiles as shown by cross-adaptation experiments, these OSNs will not be the types that convey sex-pheromone-specific information to the antennal lobe.

Keywords Asian Longhorned beetle · *Anoplophora glabripennis* · Aggregation-sex pheromone · Single sensillum recordings · Olfactory sensory neurons · OSN · Neurophysiology · Plant volatiles · 4-(n-heptyloxy) butan-1-ol · 4-(n-heptyloxy) butanal · Action potential amplitudes · Action potential frequency · Spike amplitudes · Spike frequency

Introduction

The Asian longhorned beetle (ALB), *Anoplophora glabripennis* (Cerambycidae: Lamiinae) is a serious introduced pest of hardwood trees in North America. It is known to attack nearly 50 species of apparently healthy trees, but has preferences for those in the genera *Acer, Fraxinus, Populus, Salix* and *Ulmus* (Gao and Li 2001; Hu et al. 2009; Luo and Li 1999). It is native to China, where it is a serious pest in poplar plantations. In the U.S., several infestations have occurred since it was first discovered in 1996 in Brooklyn, N.Y. (Cavey et al. 1998; Haack et al. 1997), including in the greater New York City area, Massachusetts, Illinois, New Jersey, and Ohio (Dodds and Orwig 2011). It has been found to attack many *Acer* species

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(Haack et al. 2010) in areas where it has invaded, with the invasions apparently being due to transport of larvae and pupae hidden in wood pallets on container vessels arriving from Asia.

The male-produced aggregation-sex pheromone (sensu Cardé 2014) of *A. glabripennis* was identified by Zhang et al. (2002) as a two-component mixture in a 1:1 ratio: an aldehyde, 4-(n-heptyloxy)butanal, hereafter called "ALB aldehyde", and an alcohol, 4-(n-heptyloxy)butan-1-ol, hereafter called "ALB alcohol". Laboratory studies, followed by field trapping experiments, showed the sex pheromone is effective in attracting adult females, and there are instances when the addition of plant volatiles to the sex pheromone blend can increase trap-catch over the male-produced pheromone alone (Nehme et al. 2009, 2010; Meng et al. 2014). Some field trapping experiments have indicated that the pheromone blend alone attracts predominately females, whereas plant volatiles with or without the pheromone will tend to attract a greater proportion of males (Meng et al. 2014).

Although much has been learned about the behavioral response specificities to male-produced aggregation-sex pheromone and female-produced sex pheromone blends of a large number of species within the many different subfamilies of the Cerambycidae (see Hanks and Millar 2016 for a review of pheromone structures and uses), there have been only a few studies that have attempted to learn about aspects of cerambycid beetle olfactory pathways. Two such recent studies focused on odorant receptors (ORs) and the possible antennal lobe glomerular targets of the corresponding olfactory sensory neurons (OSNs) they are expressed on (Mitchell et al. 2012, 2017), and the response profiles of OSNs that seem to be involved in cerambycid pheromone-related attraction (MacKay et al. 2015). Single sensillum recordings investigating OSN responses to odorants in the Cerambycidae are rare (Dyer and Seabrook 1978; Barata et al. 2002; Lopes et al. 2002; MacKay et al. 2015). Only one of these, MacKay et al. (2015), working with *Tetropium fuscum*, used pheromone components to try to determine whether there were aggregation-sex-pheromone-specific pathways originating from antennal OSNs to transmit pheromone information to the antennal lobe. We performed single sensillum recordings of OSNs on the antennae of male and female *A. glabripennis* to explore the sensory neurophysiological underpinnings of aggregation-sex pheromone-related and plant-volatile-related responses of female and male *A. glabripennis*.

Materials and Methods

Insects

x*Anoplophora glabripennis* were obtained from the quarantine rearing facility of the Kelli Hoover laboratory, Penn State University. Beetles were reared at 25 °C, 60% RH on a 16:8 light: dark photoperiod regime. The heads of adult males and females were removed, placed in 15 ml vials, and taken out of the facility to the Baker laboratory for further preparation for single sensillum recordings.

Electrophysiology

Tungsten electrodes were electrolytically sharpened using a 5% KNO_2 solution at 40 or 15 V. A constant 40 V current was applied for 5 min to sharpen a new electrode or for 30 s for an electrode that was being re-sharpened. Current was then reduced to 15 V for 2 min, while raising and lowering the electrode into the solution to form the finely tapered tip on the electrode. A manipulator was used to quickly raise and lower the electrode into and out of the solution at a rate of 1 Hz, and a maximum depth of 5 mm at the selected current. The oscillation was either performed manually, or by a motorized manipulator constructed from LEGO™ components.

To make a stable preparation for single sensillum recordings, the antenna was first cut from the insect near the base of antenna and affixed firmly to a glass microscope slide using either dental wax or tape. The antennae of male and female *A. glabripennis* have alternating black and white sections along their entire length, with dense setae causing the white sections' hue. The white setae obscure the olfactory sensilla and make it difficult to gain electrical connections, and so we recorded only from the black portions of flagellomeres where it was easier to visualize potential olfactory sensilla that we might be able to connect with. A reference (ground) electrode was inserted into the open base of the antenna via a manipulator. Once mounted and stabilized, the antenna was examined through a Nikon FN1 Eclipse microscope using both reflected and transmitted light, which provided a highly magnified (×750) view of the antennal sensilla. We recognized three main types of sensilla from which we tried to record OSN activities (Fig. 1a, b). One type was a so-called "blunt-tipped" basiconic sensillum (Fig. 1a; "Ba.1″), which we recorded from mostly on the middle flagellomeres nos. 4 through 7. A second type that we tried recording from was what we named a "sharp-tipped" basiconic sensillum (Fig. 1a, b; "Ba.2″). We found that this sensillum type was distributed all over the antenna, and despite numerous attempts, we either failed to gain electrical connections or else any OSNs residing in these sensilla were unresponsive to all of the compounds we exposed them to. A third type was what we designated as "trichoid" sensilla (Fig. 1b; "Tr.") from which we recorded most easily on the two most distal antennal flagellomeres of both males and females.

Making contacts with ALB sensilla and gaining good recordings was quite difficult, perhaps due to the hardness and small size of these sensilla. We found that recordings were not possible by forcefully penetrating the sensillum with a glass or tungsten electrode. Thus, among the small percentage of successful recordings we were able to obtain, success was gained when a tungsten recording electrode was touched to the base

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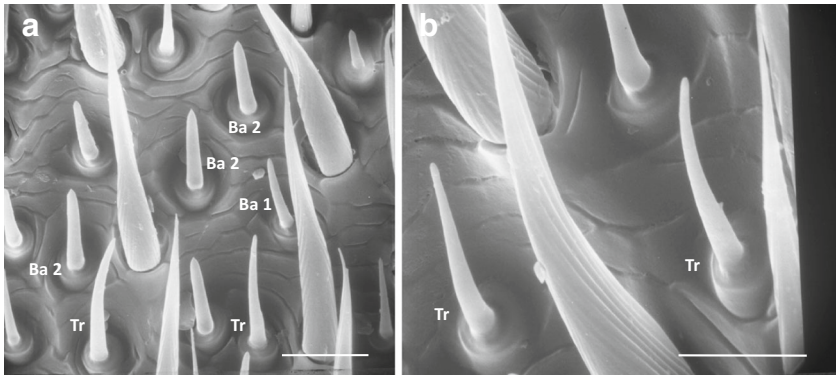

Fig. 1 Scanning electron micrographs of sensilla on the antennae of female *Anoplophora glabripennis*. **a** Trichoid and basiconic sensilla on Flagellomere 9. "Ba 2" denotes sharp-tipped type of basiconic sensillum that yielded no recordings. "Ba 1" denotes blunt-tipped basiconic sensillum. "Tr" denotes a slender, trichoid type of sensillum characterized by a slightly curved and tapered tip. **b** Trichoid sensilla ("Tr") on Flagellomere 10. Scale bar in A and B = 20 μm

of either a blunt-tipped basiconic or trichoid sensillum and adding a slight amount of pressure to apparently barely puncture it using a Narishige hydraulic micromanipulator.

Single sensillum recordings of OSNs were made using either of two systems. One system consisted of a Syntech (Buchenbach, Germany) 10X universal AC/DC probe followed by a Syntech INR2 50× AC-coupled amplifier. This signal was fed into a Hum Bug powerline interference canceller (Quest Scientific, Vancouver, Canada) and digitized using a Syntech IDAC-4-USB. Syntech Autospike v 1.3 running on a 32 bit windows XP laptop that was used to capture and display recordings obtained from the IDAC-4-USB. The second system consisted of a custom DC-coupled preamplifier and digitizer assembled in one unit. Data were acquired at a rate of 10 kHz at 18 bit resolution. The unit was connected via USB to a PC running Windows XP where data were stored. A single-pole highpass filter with a − 3 dB frequency of 100 Hz was applied to the data in software.

Odorants and Stimulus Delivery

We were primarily interested in seeing if there were any OSNs that were responsive to either of the two sex pheromone components of this species. We used a limited panel of odorants from different chemical classes to assess the specificity of any pheromone-component-responsive OSNs, as well as the responsiveness of other types of OSNs. The following compounds were used in our odorant panel, which we chose to encompass as many broad classes of volatile compounds as we could think of, while limiting the number of compounds due to the anticipated brevity of recording time stemming from the fragility of the electrophysiological connection with the OSNs. We knew that the volatiles from some of these classes did not necessarily have relevance with regard to tree or plant volatiles, but nevertheless might be informative as to the response profiles of some OSNs. The two *A. glabripennis* aggregation-sex pheromone components, 4-(n-heptyloxy) butan-1-ol and 4-(n-heptyloxy) butanal (purity 99 and 97%, respectively) were purchased from Bedoukian Research Inc. (Danbury, CT, USA). Geraniol and eugenol were purchased from Tokyo Chemical Industry Co., Ltd. (Japan) and were 96 and 98% pure, respectively. (*E*)-β-ocimene and indole were purchased from Fluka Chemical Corporation (Switzerland) and were 97% pure. Citronellal was purchased from Acros Organics B.V.B.A. (Belgium) and was 93% pure. Compounds purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), with their percent purities, were as follows: limonene (97%), β-myrcene (96%), 1-octene-3-ol (98%), linalool (97%), α-pinene (98%), linalool (99%), nerol (97%), 1-hexanol (98%), 1-heptanol (98%), 1-octanol (99%), citral (95%), β-Caryophyllene (98.5%), (*Z*)-3-hexenol (98%), benzyl alcohol (99.8%), α-terpineol (96%), nerolidol (97%), dipropyl disulfide (98%), m-cresol (95%), (*E*)-β-farnesene (> 90% "analytical standard") and allyl isothiocyanate (95%). Acetic acid (100% purity) was purchased from J.T. Baker, Inc. We occasionally included in the odorant panel a mixture of farnesene isomers ("Kosher grade"; Sigma-Aldrich Corporation) that included 21% (*E*)-β-farnesene and from 10 to 30% of other farnesenes.

The samples of (*E,E*)- and (*Z,E*)-α-farnesene isomers used in this study came from the Damon Crook laboratory at USDA/APHIS and had the following purities. The sample of (*E,E*)- α-farnesene was (3*E*,6*E*)-α-farnesene that originated from the Jocelyn Millar laboratory at the University of California, Riverside and was greater than 86% pure according to gas chromatographic analysis (Crook et al. 2014). Hereafter this sample will be referred to as "pure" (3*E*,6*E*)-α-farnesene. The sample of (*Z,E*)-α-farnesene that we used was (3*Z*,6*E*)-α-farnesene that originated from the Natural Resources Canadian Forest Service laboratory and was greater than 90% pure (Crook et al. 2014).

Ten milligrams of neat samples of each of the odorants as measured by volume (10 μl), were dissolved in 1 ml hexane and diluted in 1 ml hexane in 10-fold steps. Aliquots of 10 μl of each diluted compound were dispensed onto a filter paper strip (Whatman; 0.2 X 1.5 cm) such that odor cartridges

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contained loadings of 0.1 μg, 1 μg, 10 μg, and 100 μg of an odorant. The filter paper strips were inserted into a Pasteur pipette (15 cm long) to create the odor cartridges. A constant airflow of charcoal-purified, humidified air was passed across the antennae through a glass tube (10 mm diameter) during the experiments. Odorants were delivered into this constant air stream via the Pasteur pipette, the tip of which was inserted through a small hole in the glass tube, 11 cm away from its end. A stimulus flow controller (Syntech; Germany) pulsed a 40 ml/s air stream through the cartridge for 0.3 s, effectively delivering volatiles from the odor cartridge into the air stream and onto the antenna. Odor cartridges were capped with Eppendorf pipette tips when not in use and similarly capped and stored in a freezer at −20 °C between uses. All cartridges were reloaded and replaced after approximately three days of exposure to room temperature.

For interrogation of OSNs in single sensilla using single puffs of odorants, a good contact with a sensillum was first made and then puffs of odorants from the panel were performed in random order in order to determine the differential responsiveness of the OSNs housed in the sensillum. Subsequent trials involving each sensillum depended on these initial responses. For instance, when the type of sensillum we contacted dictated that we needed to perform a dose-response series, the order of presentation of the odorants was always from lowest to highest cartridge loading. When the initial responses indicated that we had an opportunity to perform cross-adaptation trials, we then set about trying to perform such trials.

Cross-Adaptation

For certain subsets of OSNs that we recorded from, we conducted cross-adaptation studies to determine if two different co-compartmentalized OSNs were responding to two odorants. Two odor cartridges were connected to the two stimulus delivery ports of the Syntech flow-controller. The cartridges contained loadings of the volatiles that had elicited moderate frequencies of action potentials, between 40 and 60 spikes/s, for each of the compounds in preliminary dose-response trials. For cross-adaptation involving basiconic sensillar OSNs, the cartridges thus contained 100 μg loadings of either of the two ALB pheromone components in the first cartridge, and 10 μg of either (*E*)-β-farnesene, eugenol, or β-caryophyllene, or 0.5 μg (*E,E*)-α-farnesene in the second cartridge. For cross-adaptation involving trichoid sensillar OSNs, the cartridges contained 100 μg loadings of either of the two ALB pheromone components in the first cartridge, and 10 μg of either geraniol or citronellal in the second cartridge. These plant-related or pheromone-related OSNs of interest were first located within various sensilla using a standard single puff of one of these odorants. Then a 0.25 s puff from the first odor cartridge was administered, followed by a 0.10 s period of clean air, and then a 0.25 s puff from the second odor cartridge.

Analysis of Action Potentials

Single-Puffed Stimuli

For series of recordings using single-puffed stimuli, we determined the responsiveness of the OSNs to the odorants they were exposed to by counting the number of spikes present during a 0.5 s pre-stimulus period and subtracting this background frequency from the frequency elicited during a 0.5 s post-stimulus delivery period. This latter number was then doubled to report the spike frequency on a spikes-per-second basis. Mean spike frequency (± S.D.) was calculated for each odorant for the recordings taken on different sensilla.

Cross-Adaptation Trials

To differentiate between possibly two or more different OSNs' responses from within a single sensillum, we used an automated spike-sorting program (SAPID, Smith et al. 1990). Templates for each of the two action potential amplitudes and waveforms occurring within the sensillum were formed by the program and further modified by the user after inspecting the temporal occurrence of the spikes that had been sorted according to the two templates. Following this procedure, the templates were finalized and the SAPID program sorted the spikes according to the template; then the time of occurrence of the two different types of spikes was compared against the timing of the two different stimulus deliveries.

Statistical comparisons of spike amplitudes in response to the two odorant stimuli used in different cross-adaptation trials were made by first finding the mean amplitude of the first five spikes in response to each odorant in these paired stimulations. An overall mean (± S.D.) of these individual mean spike amplitudes was then calculated for each odorant used in a particular odorant cross-adaptation comparison. In some trials (e.g. those in Fig. 3), paired T-tests using Minitab were then performed on the mean spike amplitudes of OSNs in these paired trials. When there was a significant difference in spike amplitudes in response to the two stimuli, it was concluded that two different OSNs were responding to the two different odorants. When no significant difference in spike amplitude occurred, it was concluded that there was a single OSN responding to each of the two stimuli.

In other trials (e.g. those in Tables 1 and 2), the mean ratios of amplitudes occurring in response to the two stimuli were first calculated for the OSNs on a sensillum-by-sensillum basis. Means (± S.D.) of these individual sensillar ratios were then calculated for each of the different pairs of odorants used.

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Results

We were able to gain connections with 408 sensilla on male and female antennal sensilla over the course of this study. However, the majority of these were either very short-lived for unknown reasons stemming from the OSN suddenly becoming unresponsive, or so noisy from the contact becoming poor and not being able to be corrected, that they could not be used for data analysis. Another portion of these contacts yielded no responses to any odorants that were tried, and so the connection was abandoned in favor of finding a different sensillum nearby. Thus, we were able to get usable data from OSNs housed in 71 sensilla during this study. In nearly all cases during series when we were attempting to interrogate OSNs for their responsiveness to odorants across the entire panel of odorants we had selected, only a portion of the panel could be tested before the contact with the sensillum was lost.

Terminal Antennal Flagellomeres

OSNs Tuned to ALB Aldehyde and ALB Alcohol in Sensilla on Terminal Flagellomeres

In recording from the very narrow, delicate, slightly curved and smoothly tapered sensilla we are calling "trichoid" sensilla (Fig. 1b) that we sampled here only on the terminal flagellomeres of both male and female antennae, we found OSNs exhibiting large amplitude action potentials (spikes) responsive to both the ALB aldehyde and ALB alcohol pheromone components (Fig. 2). We found these OSNs in recordings from 18 female and six male *A. glabripennis* antennae. In these sensilla, there were also smaller-spike-amplitude OSNs co-located with the pheromone-component-responsive OSNs, and these companion OSNs responded to a variety of general odorants, most often being either geraniol and/or citronellal (Fig. 2).

Cross-adaptation experiments showed clearly that the larger-spiking pheromone-component-tuned OSNs were different from those responding to plant-related odorants, which always displayed a smaller spike amplitude (Figs. 2 and 3). It did not matter whether the ALB alcohol or ALB aldehyde was puffed first or second in the cross-adaptation regime. The pheromone-component-tuned OSN displayed larger spikes in every case than the plant-volatile-tuned OSN regardless of the order of presentation (Fig. 3), with no apparent adaptation caused by the first compound in the sequence.

Cross-adaptation studies using the ALB alcohol and ALB aldehyde against each other showed that in every case the ALB aldehyde and alcohol stimulated the same large-spiking OSN in each of the trichoid sensilla we recorded from (Fig. 3). The large-spiking OSN first stimulated by the ALB alcohol was followed by the same large-spiking OSN stimulated by the ALB aldehyde, and *vice-versa* (Figs. 3 and 4). These OSNs appeared to be slightly more responsive to the alcohol compared to the aldehyde, because the spike frequency in response to a puff of ALB aldehyde that followed the puff of the ALB alcohol was more likely to be reduced or even adapted compared to when the alcohol followed the aldehyde. In five of the eight sensilla that we were able to record from in cross-adaptation trials with pheromone components, the response to the second puff was negligible in terms of change in spike frequency and with no change in spike amplitude. Thus the first-puffed pheromone component adapted the OSN so it did not respond to the second component, showing that the same OSN was tuned to both components. In these cases amplitudes to the second puff could not be discerned from those to the first puff and were not measured.

Dose-response series performed on this type of OSN indicated that the OSNs of males and females increased their spike frequencies similarly in response to increasing dosages of both the ALB alcohol and ALB aldehyde (Fig. 5). The spike frequencies from male OSNs in response to the higher doses of ALB aldehyde appeared not to reach as high a level as those of females, or as they did in response to the ALB alcohol, but this may be due to a small sample size for male OSNs in these trials.

The smaller-spiking, companion OSN in these sensilla was variously responsive to a variety of plant-related odorants, including geraniol, citronellal, limonene, nerol, 1-heptanol, 1-octanol, β-myrcene and citral (Fig. 6). Spike frequencies in response to geraniol and citronellal were consistently higher than those of most of the other odorants. There was inconsistency in the odorants we were able to test in this large panel before connections were lost, because with each connection the order of testing was randomized. However, among our findings smaller-spiking OSNs were in every case responsive to geraniol, and there was 80–100% cross-responsiveness to limonene, citronellal, citral, and 1-octanol. There were lower incidences of responsiveness to (*E*)-β-farnesene, (*E,E*)-α-farnesene ("pure"), nerol, heptanol, geranyl acetate, and (+)-carvone. Odorants that were tried on three or more of these OSNs that never evoked a response were (*Z,E*)- α-farnesene, β-caryophyllene, 1-hexanol, (*Z*)-3-hexenol, 1-octen-3-ol, benzyl alcohol, linalool, α-terpineol, (*E*)-β-ocimene, α-pinene, nerolidol, indole, acetic acid, dipropyldisulfide, allyl isothiocyanate, and m-cresol.

Middle Antennal Flagellomeres

Unlike the trichoid sensilla that housed both a large- and smaller-amplitude-spiking OSN in the basiconic sensilla we recorded from, we found evidence for only a single, large-spiking OSN that was responsive to various plant-related odorants. Even considering background firing, there was never any other type of spike train visible from possible smaller-spiking OSNs in these recordings, and the single type of large-

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Table 1 Cross-stimulation of OSNs in basiconic sensilla (Type "Ba 1") responsive to (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, and ALB aldehyde. Dosages used in odor cartridges were 100 μg, 10 μg, and 0.5 μg for the ALB aldehyde, (*E*)-β-farnesene, and (*E*,*E*)-α-farnesene, respectively

Spike Amplitude 1 (mV)	Spike Amplitude 2 (mV)	Mean Ratio (±SD) ALB-Ald-to-Plant Vol. Spikes
1st (*E*)-β-farnesene	2nd ALB aldehyde	
0.35	0.37	1.01 ± 0.02
0.40	(adapted)	(1/4 were adapted)
1st ALB aldehyde	2nd (*E*)-β-farnesene	
1.01	1.01	
1.29	1.26	
1st (*E*,*E*)-α-farnesene	2nd ALB aldehyde	
0.63	(adapted)	1.04 ± 0.04
0.61	(adapted)	(2/4 were adapted)
1st ALB aldehyde	2nd (*E*,*E*)-α-farnesene	
1.32	1.23	
1.24	1.23	
1st (*E*)-β-farnesene	2nd (*E*,*E*)-α-farnesene	
1.29	(adapted)	(2/2 were adapted)
1st (*E*,*E*)-α-farnesene	2nd (*E*)-β-farnesene	
0.59	(adapted)	

spiking OSN that was present was optimally responsive to somewhat different sets of odorants depending on which sensillum was being contacted by the electrode.

OSNs Responsive to Farnesenes and ALB Aldehyde

One common group of OSNs in blunt-tipped basiconic sensilla, mostly recorded from on the middle-antennal sensilla flagellomeres 4 through 7 of male and female ALB, responded to (*E*,*E*)-α-farnesene ("mix" and "pure"), (*E*)-β-farnesene, and to the ALB aldehyde pheromone component in various combinations. When this type of OSN was found, in none of these recordings was it responsive to the ALB alcohol pheromone component. There were six sensilla of this type that housed an OSN that responded to all three compounds (Fig. 7). Another two of these sensilla housed an OSN that responded to both (*E*)-β-farnesene and (*E*,*E*)-α-farnesene, but not to the ALB aldehyde, and another 12 of this sensillar type housed an OSN responding only to (*E*)-β-farnesene, but not to (*E*,*E*)-α-farnesene or the ALB aldehyde (not shown).

Cross-adaptation studies showed that in the instances where all three of these compounds stimulated an OSN, it was in fact the same OSN that responded with similar spike sizes to two or three of these compounds (Fig. 7; Table 1). In the small number of cases of cross-stimulation that we were able to conduct, either the response to the second compound was negligible, i.e., completely adapted (no increase in spike frequency) following the puff of the first compound (Fig. 7d), or else the spike amplitudes of the response to the second compound were not significantly different from the amplitudes in response to the first compound (Figs. 7 A–C; Table 1). This type of OSN was thus not tuned specifically to the ALB aldehyde pheromone component, but rather its response seems to be related to the OSN's response profile as being primarily sensitive to both (*E*)-β-farnesene and (*E*,*E*)-α-farnesene. This type of OSN would not report ALB-aldehyde-pheromone-component-specific information to its glomerulus in the antennal lobe due to its co-responsiveness to plant-related compounds.

OSNs Responsive to β-Caryophyllene, Eugenol, and ALB Alcohol

Another type of sensillum that we found in recording from the blunt-tipped basiconic sensilla on antennal flagellomeres 4 through 7 contained OSNs primarily responsive to both eugenol and β-caryophyllene. There were other sensilla in this group that housed OSNs that responded to these two compounds but also to the ALB alcohol (Fig. 8). In no cases did these eugenol-β-caryophyllene-ALB alcohol-responding OSNs show any activity in response to the ALB aldehyde pheromone component. There were 13 sensilla with OSNs responding only to eugenol and β-caryophyllene (data not shown), and ten sensilla containing OSNs that responded to both these compounds plus the ALB alcohol (Fig. 8).

Cross-adaptation studies showed that in the instances where all three of these compounds stimulated such an OSN, it was in fact the same OSN that was responding to all three compounds. In most of these cases of cross-stimulation, the response to the second compound was completely adapted following the puff of the first compound (Fig. 8, Table 2). In the relatively few cases in which there was no adaptation, the spike amplitudes of OSN responding to the second compound were not significantly different from the amplitudes in response to the first compound (Table 2). Thus this type of

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Table 2 Cross-stimulation of OSNs in basiconic sensilla (Type "Ba 1") responsive to β-caryophyllene, eugenol, and ALB alcohol. Dosages used in odor cartridges were 100 μg, 10 μg, and 10 μg for the ALB alcohol, eugenol, and β-caryophyllene, respectively

Spike amplitude 1 (mV)	Spike Amplitude 2 (mV)	Mean spike-size ratio (±SD ALB alcohol to plant Vols. Spikes
1st β-caryophyllene	2nd ALB alcohol	
0.39	(adapted)	1.02 ± 0.02 (N = 4)
0.75	(adapted)	(6/10 were adapted)
0.33	(adapted)	
0.87	(adapted)	
0.79	(adapted)	
1st ALB alcohol	2nd β-caryophyllene	
0.76	0.75	
0.73	0.70	
0.67	0.67	
0.71	(adapted)	
1.47	1.44	
1.39	(adapted)	
1st Eugenol	2nd ALB alcohol	
0.45	(adapted)	0.99 ± 0.06 (*N* = 6)
0.22	(adapted)	(7/13 were adapted)
0.68	0.61	
0.65	(adapted)	
0.81	(adapted)	
1.27	1.30	
1.15	(adapted)	
1st ALB alcohol	2nd Eugenol	
0.83	0.78	
0.60	0.64	
0.70	0.69	
0.68	(adapted)	
1.33	1.31	
0.79	(adapted)	
1st β-caryophyllene	2nd Eugenol	
0.41	(adapted)	1.03 ± 0.05 (*N* = 2)
0.62	0.62	(10/12 were adapted)
0.94	(adapted)	
0.71	(adapted)	
0.81	(adapted)	
1.14	(adapted)	
1st Eugenol	2nd β-caryophyllene	
0.36	(adapted)	
0.22	(adapted)	
0.84	(adapted)	
0.69	(adapted)	
1.33	1.25	
1.18	(adapted)	

OSN is not tuned specifically to the ALB alcohol pheromone component and therefore would not relay pheromone-component-specific information to its glomerulus due to its co-responsiveness to the plant-related compounds eugenol and β-caryophyllene.

Discussion

Our results demonstrate that there are OSNs in trichoid sensilla on the terminal antennal flagellomeres of both female and male *A. glabripennis* that respond to either of the two long-

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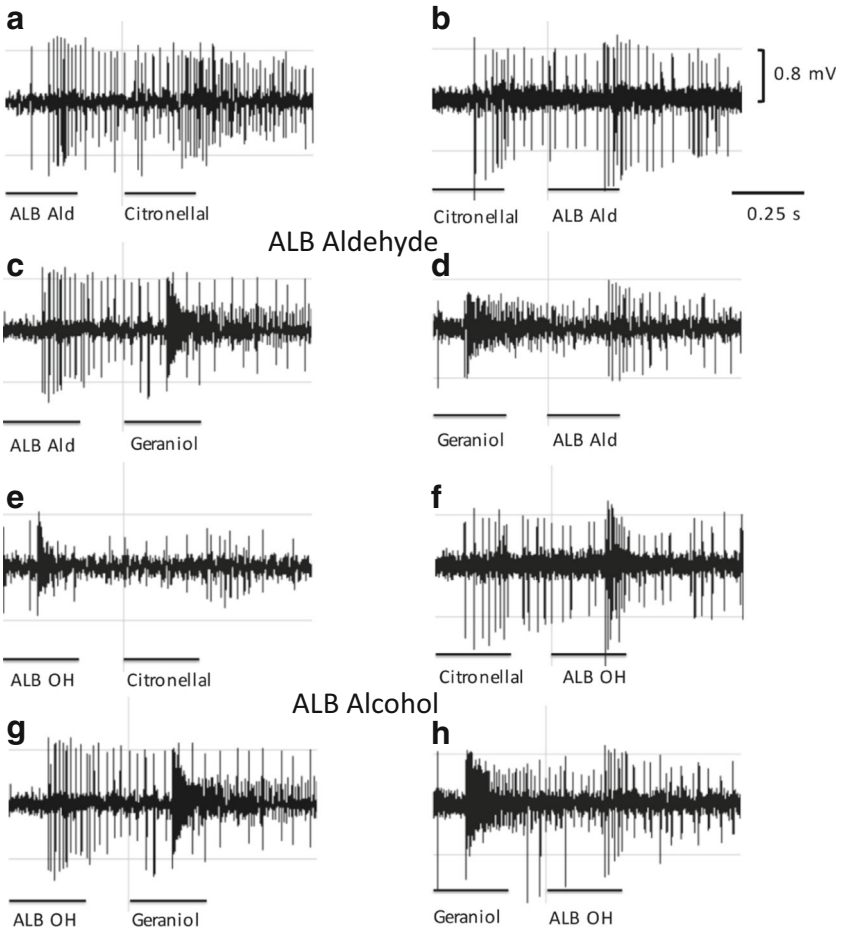

Fig. 2 **a-d.** Spike trains recorded from OSNs in *Anoplophora glabripennis* trichoid sensilla in response to successive puffs of the ALB aldehyde pheromone component and different plant volatiles showing a larger spiking OSN responding to the pheromone components regardless of order of stimulation. **a, b** The ALB aldehyde pheromone component and citronellal. **c, d** The ALB aldehyde pheromone component and geraniol. **e-h** Spike trains recorded from OSNs in *A. glabripennis* trichoid sensilla in response to successive puffs of the ALB alcohol pheromone component and different plant volatiles showing a larger spiking OSN responding to the pheromone components regardless of order of stimulation. **e, f** The ALB alcohol pheromone component and citronellal. **g, h** The ALB alcohol pheromone component and geraniol. Vertical bracket (upper right) represents 0.8 mV in all tracings. Time-scale bars represent 0.25 s

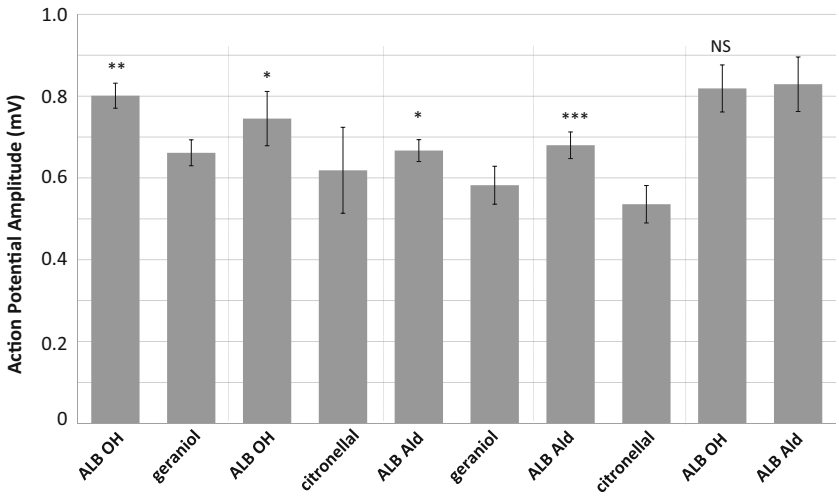

Fig. 3 Mean amplitudes (± S.E.) of action potentials recorded from *Anoplophora glabripennis* trichoid sensilla in response to successive puffs of the two ALB pheromone components (100 μg loadings of each) and either citronellal or geraniol (10 μg loadings of each). Successive puffs of the ALB aldehyde and alcohol (far right) are also shown for comparison. OSNs always exhibiting the larger amplitude spikes respond to both the aldehyde and the alcohol pheromone components, and the co-compartmentalized OSNs exhibiting smaller amplitude spikes respond to the two plant-related compounds, citronellal and geraniol. Data from successive puffs, regardless of order (plant volatile or pheromone component puffed either first or second), were merged. * = $P < 0.05$; ** = $P < 0.01$; *** $P < 0.001$ according to paired T-tests between odorant pairs. $N = 9$, 4, 10, 8, and 6 for ALB OH/geraniol, ALB OH/citronellal, ALB Ald/geraniol, ALB aldehyde/citronellal and ALB Ald/ALB OH pairs, respectively

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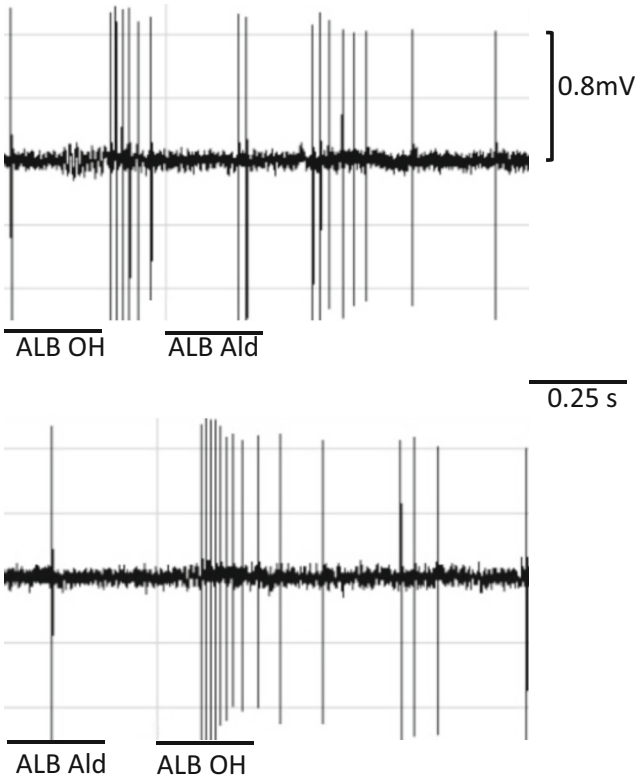

Fig. 4 Spike trains recorded from the large spiking OSN in an *Anoplophora glabripennis* trichoid sensillum in response to successive puffs of the ALB aldehyde and ALB alcohol pheromone components (100 μg loadings of each) showing that this larger spiking OSN responds to both pheromone components. Vertical bracket represents 0.8 mV. Time-scale bars represent 0.25 s

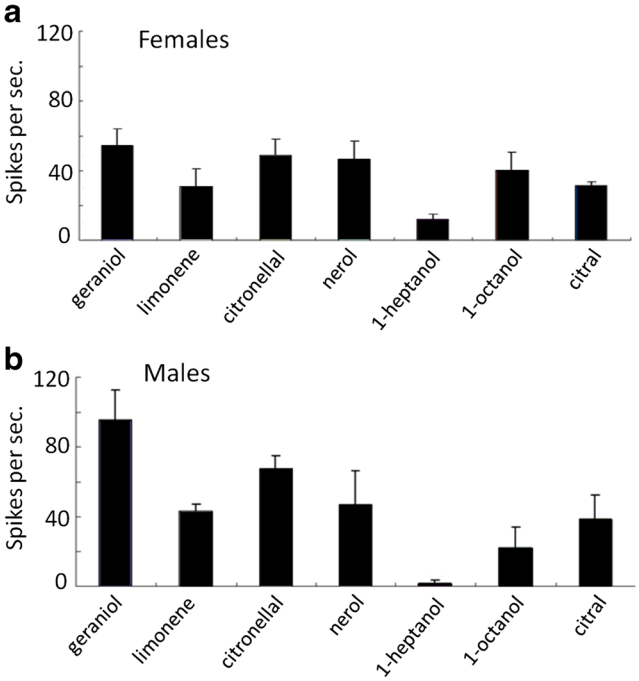

Fig. 6 Mean (± S.E.) action potential frequencies of *Anoplophora glabripennis* female (top) and male (bottom) smaller-spiking OSNs housed in trichoid sensilla responding to a 10 μg doses of plant-related volatiles. For females, $N = 4$, 3, 3, 2, 3, 3, and 2 for geraniol, limonene, citronellal, nerol, 1-heptanol, 1-octanol, and citral, respectively. For males, $N = 4$, 4, 3, 3, 2, 3, and 3 for geraniol, limonene, citronellal, nerol, 1-heptanol, 1-octanol, and citral, respectively

range aggregation-sex pheromone components of this species (Zhang et al. 2002) and not to other compounds we have tested. These OSNs display a larger-amplitude action potential than a second, smaller-spiking type of the OSN with which they are co-compartmentalized in these sensilla. This type of sex-pheromone-component-responsive OSN thus far appears to be narrowly responsive to the two sex pheromone components and thus will be able to convey aggregation-sex-pheromone-related information to higher olfactory centers when either of these aggregation-sex pheromone components wafts over the antenna. This information will travel along distinct olfactory pathways to the antennal lobe that are

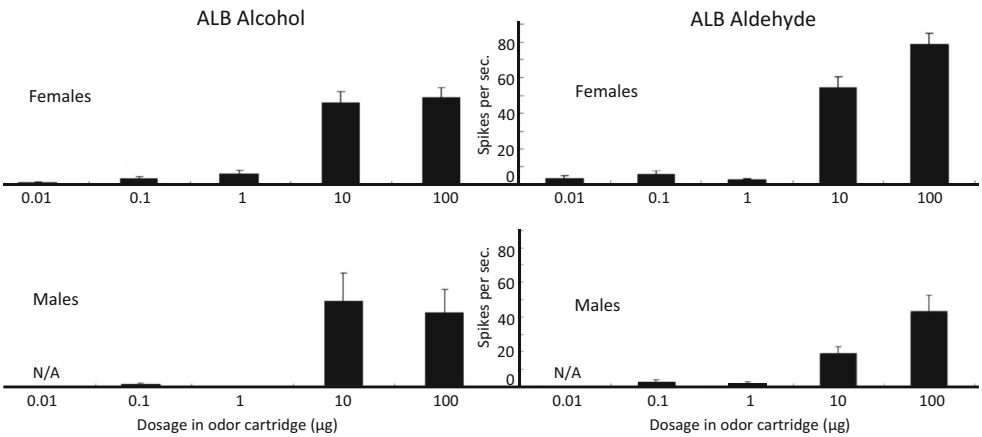

Fig. 5 Mean (± S.E.) action potential frequencies of *Anoplophora glabripennis* female (top) and male (bottom) recorded from the large-spiking OSNs housed in trichoid sensilla responding to a dose-response series of either the ALB alcohol pheromone component (left) or ALB aldehyde component (right). For the ALB alcohol for females, $N = 9$, 15, 14, 15, and 12 for the 0.01, 0.1, 1, 10, and 100 μg doses, respectively; for males, $N = 3$ for all dosages. For the ALB aldehyde for females, N = 9, 15, 15, 15, and 14 for the 0.01, 0.1, 1, 10, and 100 μg doses, respectively; for males, N = 3 for all dosages

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unrelated to information pathways about plant volatiles, because thus far we have not found these OSNs to be responsive to any of the plant-related compounds that we have tested. In a study of male and female antennal lobe glomeruli of *A. glabripennis,* an enlarged glomerulus was found in both males and females (Mitchell et al. 2017) at the entrance of the antennal nerve to the antennal lobe. This may be the glomerulus that receives pheromone component information from this type of OSN.

It is interesting that this type of large-spiking OSN is not specifically tuned to either the ALB alcohol or the ALB aldehyde, although it seems to be slightly more responsive to the ALB alcohol than the aldehyde. Further recordings may reveal OSNs that have such pheromone-component specificity for either the ALB alcohol or aldehyde, but not both. In our limited sampling thus far, we found this type of OSN on both male and female antennae. Its presence on both sexes could be because as with male-emitted sex pheromones of many other groups of insects, the male-emitted *A. glabripennis* aggregation-sex pheromone attracts not only females, but also males, who are likely opportunistically attracted to be present when females arrive at the pheromone-emitting male in order to intercept arriving females. Thus it behooves both males and females to have OSNs that are responsive to sources of this pheromone in order to gain matings.

The smaller-spiking type of OSN co-compartmentalized with the large-spiking pheromone-component-responding OSN interestingly did not respond to (*E*)-β-farnesene or (*E,E*)-α-farnesene. The latter compound has been implicated as a third pheromone component of *A. glabripennis* (Crook et al. 2014) and it was logical to expect that an OSN tuned to this compound might be co-located in the same sensillum as the ALB alcohol- or ALB aldehyde-tuned OSN. However, we did find OSNs that were fairly specifically responsive to (*E,E*)-α-farnesene in basiconic sensilla on flagellomeres 4–7, and these might possibly be responsible for the increased attraction seen to this compound when co-emitted with the ALB alcohol and aldehyde (Crook et al. 2014). It is notable that we found this type of (*E,E*)-α-farnesene-responsive OSN to be sometimes also responsive to the ALB aldehyde pheromone component, but not the ALB alcohol. In these cases the co-responsiveness to ALB aldehyde might explain the pheromone-contributing activity of (*E,E*)-α-farnesene found by Crook et al. (2014) because this might be a pathway by which either the ALB aldehyde or (*E,E*)-α-farnesene can contribute to pheromone-mediated behavior in conjunction with the ALB alcohol that uses its own pathway provided by the large-spiking OSNs found in trichoid sensilla.

In the mid-antennal flagellomeres we also found a type of OSN that responded to eugenol, β-caryophyllene, and the ALB alcohol pheromone component (Table 2; Fig. 8) and not to other compounds we tested. The co-responsiveness to the ALB alcohol by these OSNs will not convey pheromone-specific information to higher centers in these cases because the OSNs are also responsive to these different plant volatiles. Thus, due to this non-pheromone-component-specific tuning, the OSNs will arborize in a glomerulus that is different from that to which ALB-alcohol-pheromone-component-specific OSNs in trichoid sensilla project their axons.

Our results with *A. glabripennis* are similar in some ways to those of MacKay et al. (2015) with *T. fuscum*, a cerambycid species from the subfamily Spondylidinae. They found a large number of OSNs that were responsive to this species' main pheromone component, fuscumol, but only approximately half of these OSNs were specifically responsive only to fuscumol. The rest were responsive to fuscumol plus various plant-related odorants. In our study we found OSNs in trichoid sensilla of *A. glabripennis* that were specifically responsive to the ALB alcohol and ALB aldehyde, but in the basiconic sensilla we recorded from there was another set of OSNs that responded to either the ALB alcohol or aldehyde as well as to plant-related odorants, most commonly eugenol, β-caryophyllene, (*E*)-β-farnesene or (*E,E*)-α-farnesene. As in the MacKay et al. (2015) study, we found OSNs specifically tuned to the ALB pheromone components on male as well as female antennae. Their sensitivity to either component did not appear to differ between the sexes.

In the MacKay et al. study, it was not clear from which types of sensilla they found fuscumol-sensitive OSNs, although in a previous paper MacKay et al. (2014) had characterized trichoid, "basiconic 1", and "basiconic 2" sensilla along the antennae in addition to other sensillar types. For *A. glabripennis*, we found pheromone-component-specific OSNs in trichoid sensilla and also OSNs in basiconic sensilla that were co-responsive to the ALB aldehyde plus the farnesenes, or to the ALB alcohol plus eugenol and (*E*)- β-caryophyllene. The trichoid sensilla from which we recorded (Fig. 1b), appeared to be slightly more smoothly tapered than the trichoid sensilla of *T. fuscum*, which in their scanning electron micrographs appeared to have a more sharpened tip than those of *A. planipennis* (MacKay et al. 2014). In contrast to our recording technique using sharpened tungsten electrodes touching the base of sensilla, MacKay et al. (2015) were able to use a cut-sensillum technique plus glass-saline electrodes to record from the sensillar tips, apparently due to the longer, more accessible sensilla on the antennae of this species. Perhaps this resulted in these authors being more likely to cut and record from the longer trichoid sensilla than from other types. The *A. glabripennis* sensilla are not very accessible for tip-cutting, lying fairly flat along the antennal surface (Fig. 1b). It would be interesting to know whether, as we found in *A. glabripennis* pheromone-specific OSNs, the *T. fuscum* pheromone-specific OSNs are more likely to be housed in trichoid sensilla than in other types.

In our study, the pheromone-component-specific OSNs found in trichoid sensilla were always the larger-spiking units

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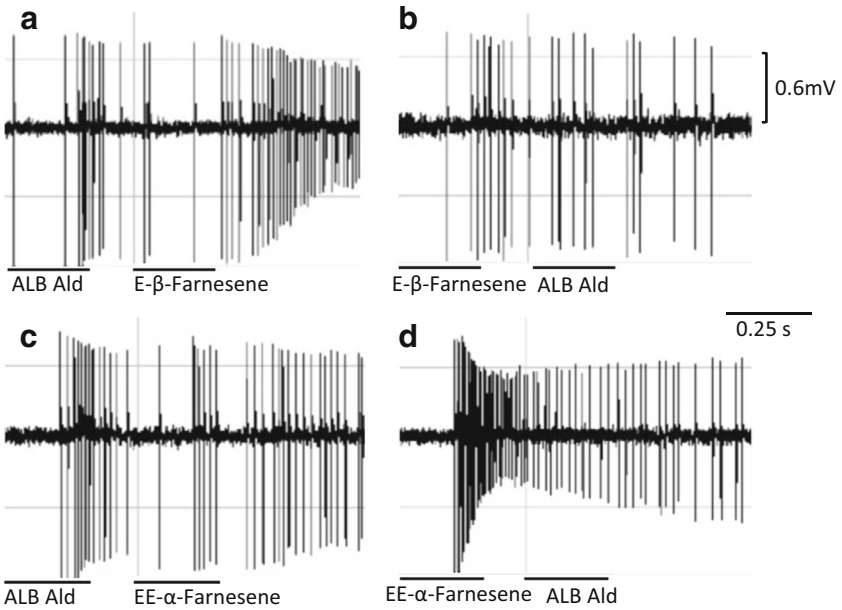

Fig. 7 Spike trains recorded from OSNs in *Anoplophora glabripennis* basiconic (Type Ba 1) sensilla that house OSNs responsive to the ALB aldehyde pheromone component plus two farnesenes, but not the ALB alcohol. Responses are shown to successive puffs of the ALB aldehyde and either (*E*)-β-farnesene (**a, b**) or (*E,E*)-α-farnesene (**c, d**). Dosages used in odor cartridges were 100 μg, 10 μg, and 0.5 μg for the ALB aldehyde, (*E*)-β-farnesene, and (*E,E*)-α-farnesene, respectively. Vertical bracket at upper right represents 0.6 mV. Time-scale bars represent 0.25 s

and plant-related odorants were the smaller-spiking type. Similar to our study for pheromone-component-specific OSNs, MacKay et al. (2015) found for *T. fuscum* that the majority (15 out of 21) of fuscumol-specific OSNs were large-spiking. The significance of this trend for large-vs.-small-spiking OSNs is unknown at present, and it is possible that more extensive sampling of *A. glabripennis* trichoid sensilla might, as for *T. fuscum*, result in finding small-spiking OSNs responsive to the ALB pheromone components as well as larger-spiking units. In many moth families in which pheromone-component-specific OSNs are co-located within the same sensilla, such as *Ostrinia nubilalis* in the Crambidae, larger-spiking OSNs are the ones that are responsive to the major pheromone component and also project to the larger of two glomeruli accepting pheromone-component-specific information from the antenna (Koutroumpa et al. 2014). In a study of antennal lobe morphology of *A. glabripennis*, Mitchell et al. (2017) described an enlarged glomerulus at the entrance to the antennal lobe in both males and females, and conjectured that this glomerulus might be receiving pheromone-component-specific inputs from antennal OSNs. If this is so, then it is reasonable to hypothesize that this glomerulus is receiving

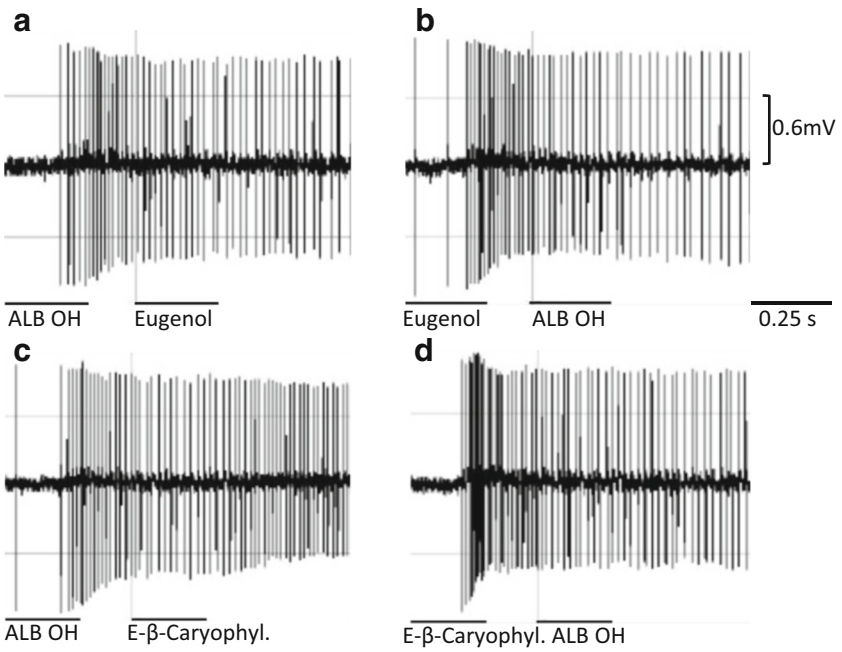

Fig. 8 Spike trains recorded from OSNs in *Anoplophora glabripennis* basiconic (Type Ba 1) sensilla that house OSNs responsive to the ALB alcohol pheromone component plus eugenol and β-caryophyllene, but not the ALB aldehyde. Responses are shown to successive puffs of the ALB alcohol pheromone component and either eugenol (**a, b**) or β-caryophyllene (**c, d**). Dosages used in odor cartridges were 100 μg, 10 μg, and 10 μg for the ALB alcohol, eugenol, and β-caryophyllene, respectively. Vertical bracket at upper right represents 0.6 mV. Time-scale bars represent 0.25 s

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excitation from the large-spiking OSNs specifically responsive to the ALB alcohol and aldehyde we recorded from in this study. Glomeruli receiving inputs from (*E,E*)-α-farnesene-plus-ALB-aldehyde-tuned OSNs or from as-yet-undiscovered ALB-aldehyde-specific OSNs could be expected to arborize in one of the smaller glomeruli found by Mitchell et al. (2017).

We did not examine trichoid sensilla in the more medial region of the antennae, and therefore we cannot say whether the distal segments house more pheromone-component-tuned OSNs than more proximal areas. Electroantennogram (EAG) studies (Hall et al. unpublished data) showed no differences in four antennal regions with regard to EAG amplitude ratios in response to the pheromone components vs. other, general odorants. Therefore, it seems likely that there are trichoid sensilla containing pheromone-component-specific OSNs in more proximal regions of the antenna as well as towards the tip. Some of the pheromone-responsive OSNs that would be contributing to EAGs, however, will include those that are housed in the basiconic sensilla that we recorded from in the mid-antennal region that were co-responsive to the pheromone plus plant-related odorants. Further exploration of the *A. glabripennis* antennae for trichoid sensillar OSNs is warranted to determine whether these pheromone-specific OSN responders are weighted toward the antennal tip or not.

It is interesting that in some of the OSNs in mid-antennal-region basiconic sensilla that were co-responsive to the ALB aldehyde and (*E,E*)-α-farnesene might explain the pheromone-component behavioral activity of both (*E,E*)-α-farnesene (Crook et al. 2014) and the ALB aldehyde. The OSNs that we found in trichoid sensilla responded to both the ALB alcohol and the ALB aldehyde, and therefore at least from this type of OSN, there can be no aldehyde- or alcohol-specific pheromone-component pathway to the antennal lobe glomeruli. Therefore this OSN type does not explain the increased behavioral activity of the blend of alcohol and aldehyde in the laboratory and field (Meng et al. 2014; Nehme et al. 2009, 2010). A separate channel to the antennal lobe is needed for aldehyde component, or else for (*E,E*)-α-farnesene, and the type of OSN in mid-region basiconic sensilla responding to both the ALB aldehyde and (*E,E*)-α-farnesene might provide such a pathway to produce heightened behavioral responses to the blend of all three components.

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