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ORIGINAL PAPER

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A comparison of responses from olfactory receptor neurons of *Heliothis subflexa* and *Heliothis virescens* to components of their sex pheromone

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Abstract Single-cell electrophysiological recordings were obtained from olfactory receptor neurons in sensilla trichodea on male antennae of the heliothine species *Heliothis subflexa* and the closely related congener *H. virescens*. A large percentage of sensilla (72% and 81%, respectively, of all sensilla sampled) contained a single odor-responsive receptor neuron tuned to the major pheromone component of both species, *Z*-11-hexadecenal. A second population of sensilla on *H. subflexa* antennae (18%) housed receptor neurons that were tuned to *Z*-9-hexadecenal but also responded with less sensitivity to Z-9-tetradecenal. A similar population of sensilla (4%) on *H. virescens* male antennae housed receptor neurons that were shown to be tuned specifically only to *Z*-9-tetradecenal, with no response to even high dosages of *Z*-9-hexadecenal. A third population of sensilla (comprising 8% and 16% of the sensilla sampled in *H. subflexa* and *H. virescens*, respectively) housed two olfactory receptor neurons, one of which was tuned to *Z*-11-hexadecenyl acetate and the other tuned to *Z*-11-hexadecenol. In *H. subflexa* the *Z*-11-hexadecenyl acetate-tuned neuron also responded to *Z*-9-tetradecenal with nearly equivalent sensitivity. The behavioral requirements of males of these two species for distinct pheromonal blends was, therefore, reflected by the subtle differences in the tuning properties of antennal olfactory receptor neurons.

Keywords *Heliothis subflexa* · *Heliothis virescens* · Olfactory receptor neurons · Sex pheromone · Single-cell recordings

Abbreviations *MGC* macroglomerular complex · *ORN* olfactory receptor neuron · *Z9–14:Ald* (*Z*)-9-tetradecenal · *Z9–16:Ald* (*Z*)-9-hexadecenal · *Z11–16:Ac* (*Z*)-11-hexadecenyl acetate · *Z11–16:Ald* (*Z*)-11-hexadecenal · *Z11–16:OH* (*Z*)-11-hexadecenol

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Introduction

Much work has been performed in recent years on the olfactory pathways of moths that are related to discrimination of sex pheromone blends (Hansson 1995; Hansson and Christensen 1998; Hansson et al. 1995; Hildebrand 1995; Hildebrand and Shepherd 1997). One group of moths that has been investigated intensively is the heliothine moths in the family Noctuidae, and the rigor of the neurophysiological studies at both the CNS and peripheral receptor neuron levels has been complemented by extensive behavioral studies that have allowed logical interpretations of the neuronal responses to be made.

Although two North American species, *Helicoverpa zea* and *H. virescens*, have been the focus of most of the neuroethological studies relating sex pheromone-mediated behavior to olfactory processing (Vetter and Baker 1983; Tumlinson et al. 1975; Vickers et al. 1991, 1998; Teal et al.1986; Sparks et al. 1979; Ramaswamy et al. 1985; Roelofs et al. 1974; Klun et al. 1979, 1980a, 1980b; Fadamiro et al. 1999; Berg et al. 1995, 1998; Almaas et al. 1991; Christensen 1997; Christensen et al. 1991, 1995; Cossé et al. 1998; Grant et al. 1989), a third

species, *H. subflexa*, has received less attention. *H. subflexa* feeds primarily on plants in the genus *Physalis* (Solanaceae) (Laster 1972) and is considered a pest of ground cherry (*Physalis angulata*) in Mexico. Its geographic range overlaps extensively with that of *H. zea* and *H. virescens* in the southern United States (Teal and Tumlinson 1997). Like *H. zea* and *H. virescens*, *H. subflexa* uses (*Z*)-11-hexadecenal (Z11–16:Ald) as its major pheromone component (Klun et al. 1982; Heath et al. 1990; Teal et al. 1981; Teal and Tumlinson 1997). However, two other compounds are essential for upwind flight and source location (Vickers 2002). One is (*Z*)-11-hexadecenol (Z11–16:OH) (Heath et al. 1990; Vickers 2002), which has been demonstrated to be antagonistic to attraction when added to the pheromone blends of *H. zea* (Quero and Baker 1999; Quero et al. 2001) and *H. virescens* (at $>3\%$ loading) (Vetter and Baker 1983).

The other vital secondary component of the *H. subflexa* sex pheromone blend, (*Z*)-9-hexadecenal (Z9–16:Ald) (Vickers 2002), is also the secondary component of the *H. zea* blend (Klun et al. 1980b; Pope et al 1984; Vetter and Baker 1984). However, after many experiments over the years, this compound has not been shown to be behaviorally active as part of the *H. virescens* blend (Vickers et al. 1991; Vetter and Baker 1983). A fourth compound known to be emitted by *H. subflexa* females, (*Z*)-11-hexadecenyl acetate (Z11–16:Ac), had no significant effect upon the responses of *H. subflexa* males when added to the three-component blend (Vickers 2002). Upwind flight of both *H. zea* and *H. virescens* males is antagonized by the presence of Z11–16:Ac when added in small amounts to their otherwise attractive pheromone blends (Vickers and Baker 1997; Quero and Baker 1999; Quero et al. 2001). *H. virescens* uses as its secondary sex pheromone component (*Z*)-9-tetradecenal (Z9–14:Ald).

The olfactory receptor neurons (ORNs) of male moths are housed within long cuticular hairs called sensilla trichodea usually in groups of two or three ORNs per hair. Each ORN sends axons out of the hair it resides in, down the antennal nerve, until the axon arborizes in knots of neuropil called glomeruli. Glomeruli are clustered together in the first olfactory integrative region of the brain called the antennal lobe. The glomeruli involved in sex pheromone olfaction are organized into a major sub-region of the antennal lobe called the macroglomerular complex (MGC). The MGC architecture of *H. zea* differs from that of *H. virescens* and *H. subflexa,* the latter two being essentially indistinguishable (Christensen et al. 1991, 1995; Berg et al. 1998, 2002; Vickers et al. 1998; Vickers and Christensen 2003). In all three species each essential component of the respective pheromone blend is represented in a single MGC glomerulus. In *H. zea* and *H. virescens* at least one glomerulus is dedicated to processing known behavioral antagonists.

Matings between *H. subflexa* and *H. virescens* produce viable hybrids (Laster 1972; Proshold et al. 1983), providing the material for potentially enlightening neuroethological studies on the evolution of heliothine olfaction. As a prelude to these studies of hybrid moths, however, we sought to perform recordings on the previously uninvestigated *H. subflexa* antennal receptor neuron system in order to begin to understand the tuning curves of the ORNs and how they relate to the MGC neuroanatomy and central neurophysiology described recently (Vickers and Christensen 2003). Berg et al. (1998) performed an informative study on peripheral ORN types in *H. virescens* with the goal of staining them with cobalt in order to determine their projection destinations in the antennal lobe of the brain. In preparing for studies of hybrid and backcross males, in addition to characterizing the ORN types of *H. subflexa*, we also needed to re-examine the *H. virescens* ORN types particularly with respect to their tuning curves (not performed by Berg et al. 1998). Definitive differences between *H. virescens* and *H. subflexa* parental ORN physiologies might then serve as specific characters for determining the inheritance and segregation of olfactory traits in hybrid and backcross males. This study therefore examines the differences in pheromone-related peripheral olfactory processing between *H. virescens* and *H. subflexa*. Any species-specific differences in the tuning properties of ORNs and their patterns of co-compartmentalization within sensilla will form the foundation for studying hybrid and backcross antennae and the ORNs present therein.

Materials and methods

Insects

A colony of *H. virescens* was maintained at Iowa State University as described in Vickers et al. (1991) on a 16:8 L:D photoperiod at 25°C, 40–50% RH. *H. subflexa* were reared under similar conditions at the University of Utah (Vickers 2002). Pupae of each species were sexed and adults separated daily to obtain individuals of known age. Pupae of *H. subflexa* were shipped overnight to Ames, Iowa, for electrophysiological investigations on the adult male moths after emergence.

Recordings from single sensilla trichodea

We used the cut-sensillum technique (Kaissling 1974; Van der Pers and Den Otter 1978) to record from the ORNs within an individual antennal sensillum. The male moth was placed inside a disposable pipette tip with the narrow end cut to allow the head to pass through. The head was immobilized with dental wax, and a Ag/AgCl wire was inserted into the abdomen to serve as a ground connection. The preparation was secured with an alligator clip, making contact with the Ag/AgCl wire, and mounted on a Syntech INR-2 Portable Recording Unit (Syntech, Hilversum, The Netherlands). The antenna was maneuvered with a micromanipulator until a single sensillum trichodeum rested on the sharpened blade of a stationary, vertically positioned tungsten knife, with the sensillum tip hanging over the edge. The tip was cut off using a horizontally oriented mobile tungsten knife placed in a second micromanipulator. The cut end was immediately contacted with a saline-filled glass micropipette containing Ag/AgCl recording electrode.

The AC signal from the recording electrode was connected to the built-in amplifier of the portable recording unit and the AC output fed into a computer. The neural activity (action potentials) was monitored by a loudspeaker and displayed on the computer. We processed the data with a PC-based signal processing software, Syntech AutoSpike version 4.0 (Syntech).

A stream of purified and humidified air continuously blew over the antenna (10 ml s^{-1}) through a 14-cm-long glass tube (8 mm ID) whose outlet was positioned 2 cm from the antenna. With a stimulus flow-controller device (SFC-2, Syntech), a 30-ms air pulse at a 15 ml s^{-1} flow rate was injected through the odor cartridge and into the airstream flushing the antenna.

Action potential frequency was calculated by counting the number of spikes occurring during the first 200 ms of the spike train initiated by a stimulus puff. For indiscernibly responding or poorly responding ORNs, spikes were counted during the same 200-ms post-stimulus interval as was used for counting spikes of responding ORNs. Action potentials from ORNs were declared to be 'larger' or 'smaller' than the other ORN housed in the same sensillum after measuring and taking a mean of the largest 10 spikes in the spike trains that occurred in response to particular compounds.

Except for sensillar spatial distribution studies in which transects across the width of the antenna were performed, recordings were made from randomly selected sensilla close to the dorsal (scale-covered) surface on the proximal one-third of the antenna (Grant et al. 1989; Cossé et al. 1998). In order to begin to determine whether different sensillar types occur in spatially consistent locations across the antenna, we sampled consecutive sensilla along a transect beginning at lateral edge where sensilla are the longest and progressing medially toward the center of the antenna where sensilla were the shortest (Figs. 1, 4). We chose flagellomere number 20 (counting from the antennal base) as the target flagellomere to perform this sampling.

Odor cartridges

Serial dilutions of Z11–16:Ald, Z9–16:Ald, Z11–16:OH, Z11–16:Ac, and Z9–14:Ald were made in HPLC-grade hexane using neat material stored in our laboratory. The purity of the compounds was >98% as determined by capillary gas chromatography-mass spectrometry (GC-MS), and the compounds were free of cross-contamination from the other pheromone components. All GC-MS analyses were performed using a Hewlett-Packard 5890 GC with a direct interface to a Hewlett-Packard 5972 mass selective detector (30-m DB-225 capillary column, electron impact, 70 eV). Serial dilutions of the compounds were made in redistilled HPLC-grade hexane, and the solutions were stored in 4-ml glass vials at −20°C.

For each of the compounds, 10 µl of a diluted solution was pipetted onto a 0.5×2.0 cm filter-paper strip held in a Pasteur pipette (15 cm long), hereafter referred to as the odor cartridge. Stimulus doses (loadings on filter paper) tested were 3, 10, 30, 100, and 300 µg, respectively. Solutions were checked by GC-MS to confirm that the amounts of respective compounds at a particular concentration were equal. Antennae were screened for different types of sensilla according to the response profiles of the ORNs housed inside by using the 10-µg cartridges and 30-ms puffs of air pulsed through the cartridge, into the airstream, and over the antenna as described above.

For dose-response profiles, stimulus compounds were selected in random order, beginning with the 3-µg odor cartridges and progressing through the increased dosages up to the 300-µg cartridges. The time period allowed to elapse between every puff was set at 30 s. At the end of each dose-response test, the antenna was stimulated with an additional 100-µg dose of either Z11–16:Ald, Z11–16:Ac, or Z9–14:Ald to verify the initial ORN activities. Response data obtained from ORNs that failed to respond a second time to test stimuli were not included in the final dose-response analyses.

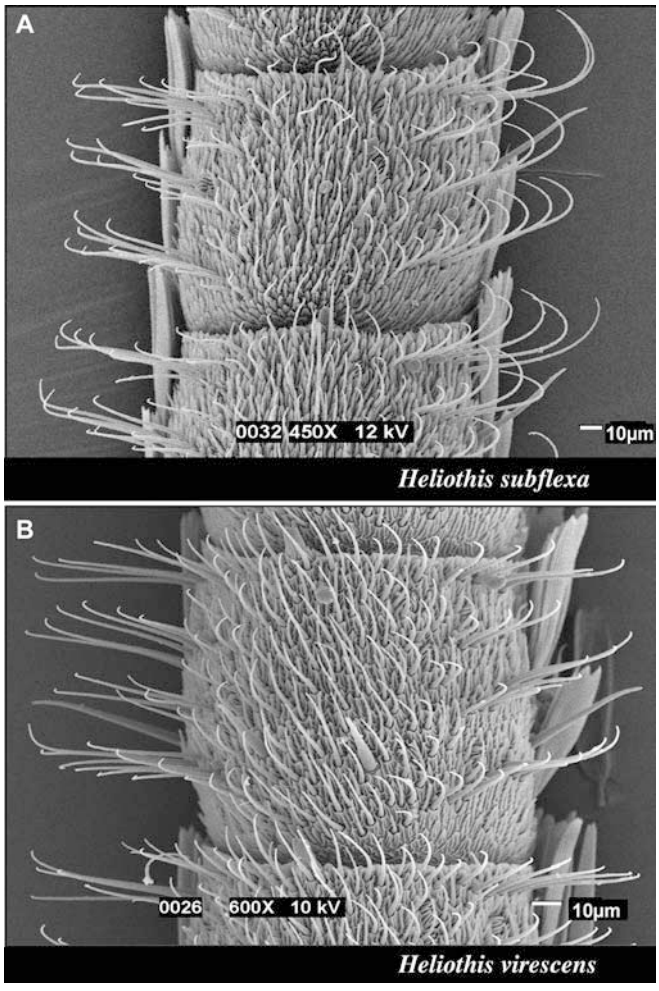


Fig. 1 Scanning electron micrograph of **A** *H. subflexa* male antenna and **B** *H. virescens* male antenna, both at approximately the 20th flagellomere from the antennal base. Scale bars 10 µm

Stimulus collections

In order to analyze the tuning of the ORNs according to the amounts emitted from the odor cartridges as opposed to the filter paper loadings in the cartridges, the compounds emitted from odor cartridges were collected in dry-ice-cooled, 25-cm-long glass collection tubes as they issued from the pipette tip. The amounts emitted, per puff, of each of the stimulus compounds used in these experiments were then quantified as described in Cossé et al. (1998).

Cross-adaptation studies

Some of our results indicated that based on the amplitude of the action potential impulses, only one ORN within a sensillum was responding to two different compounds. Other results seemed to indicate that two different action potential impulse amplitudes might be occurring, suggesting that two different types of ORN were responding to the different stimulus compounds. However, ORN impulse amplitudes are not definitive; for example, two differently tuned ORNs co-compartmentalized within the same sensillum can exhibit indiscriminable impulse amplitudes, as in European corn borer hybrids (Hansson et al. 1987).

Therefore, in order to determine more definitively how many ORNs might be contributing to the spike trains, we used a cross-adaptation technique employed in our previous work on *H. zea*

(Cossé et al. 1998). Using the stimulus flow controller, a single 50 ms puff was generated followed by an inter-stimulus interval of either 0.3 or 1 s, and then a second 50 ms stimulus from a second cartridge was generated. Depending on the type of sensillum and ORNs, the stimulus regime consisted of (1) one of the two pheromone compounds emitted from cartridge 1 followed by the same compound from cartridge 2 (self adaptation using compound 1); (2) this first pheromone compound emitted from cartridge 1, then followed by the second pheromone compound emitted from cartridge 2; (3) same as (2), but with the order of presentation reversed; and (4) compound 2 emitted from cartridge 1 followed by this same compound emitted from cartridge 2 (self adaptation using compound 2).

Fig. 2A–C Spike-train response profiles of *H. subflexa* olfactory receptor neurons (ORNs) housed within three types of sensilla trichodea we have named type A, B, and C, respectively. **A** Type A sensilla show a single odorant-responsive ORN that fires in response to (*Z*)-11-hexadecenal (Z11–16:Ald). **B** In type B sensilla, there is a single ORN that only responds to either (*Z*)-9-hexadecenal (Z9–16:Ald), or to a lesser degree to (*Z*)-9-tetradecenal (Z9–14:Ald). No other compounds evoked responses from ORNs within these sensilla. **C** In type C sensilla the same larger-amplitude spiking ORN responds to both (*Z*)-11-hexadecenyl acetate (Z11–16:Ac) and Z9–14:Ald, and a smaller-amplitude-spiking ORN responds to (*Z*)-11-hexadecenol (Z11–16:OH). For dose-response curves from these ORNs, please see Fig. 5. Responses to a blank odor cartridge (not shown) are similar in appearance to the responses to Z11–16:Ald by the ORNs in B- or C-type sensilla. Loadings in the cartridges used to evoke these spike trains were: 100 μg Z11–16:Ald, 10 μg Z9–16:Ald, 100 μg Z9–14:Ald, 10 μg Z11–16:OH, and 10 μg Z11–16:Ac, respectively

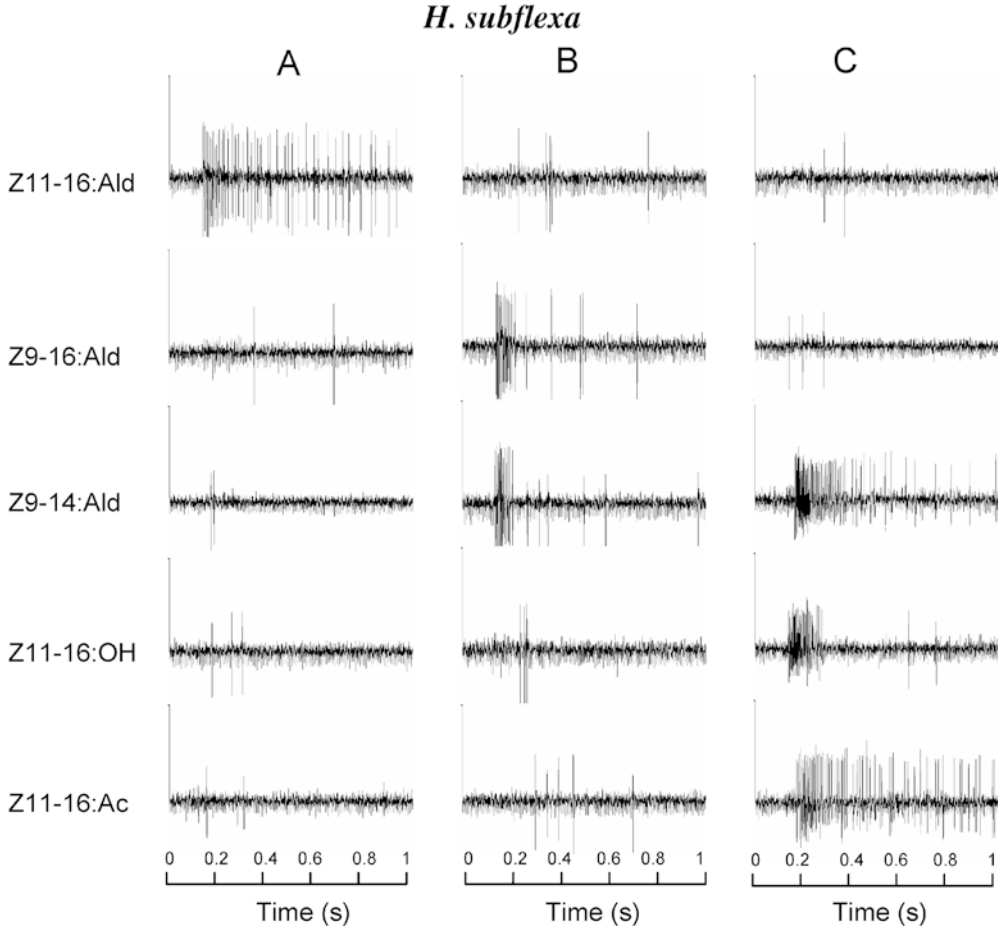
Results

H. subflexa

We recorded from 292 sensilla using 73 antennae of male *H. subflexa* (Fig. 1A). There were three distinctly different response profiles occurring in these recordings. ORNs in 72% of these sensilla (211 out of 292 sensilla) responded only to the major pheromone component, Z11–16:Ald (Fig. 2A), and exhibited no activity to any of the other pheromone-related compounds at any emitted amount.

ORNs in ca. 18% of the sensilla (53 out of 292) responded to both the secondary pheromone component of *H. subflexa*, Z9–16:Ald (Fig. 2B), as well as to Z9–14:Ald, a compound eliciting no known behavioral activity in *H. subflexa* (Vickers 2002). Cross-adaptation studies (Fig. 4A; $n = 5$ sensilla on 5 antennae) indicated that the activity in these sensilla came from a single ORN that responded to both Z9–16:Ald and Z9–14:Ald (Fig. 4 A). Dose-response curves using emitted amounts from the odor cartridges (Cossé et al. 1998) indicated that this ORN was more sensitive to Z9–16:Ald than to Z9–14:Ald (Fig. 5B).

In the third type of sensillum encountered, comprising ca. 8% of those sampled (24 out of 292), ORN responses were generated from puffs of Z11–16:OH,



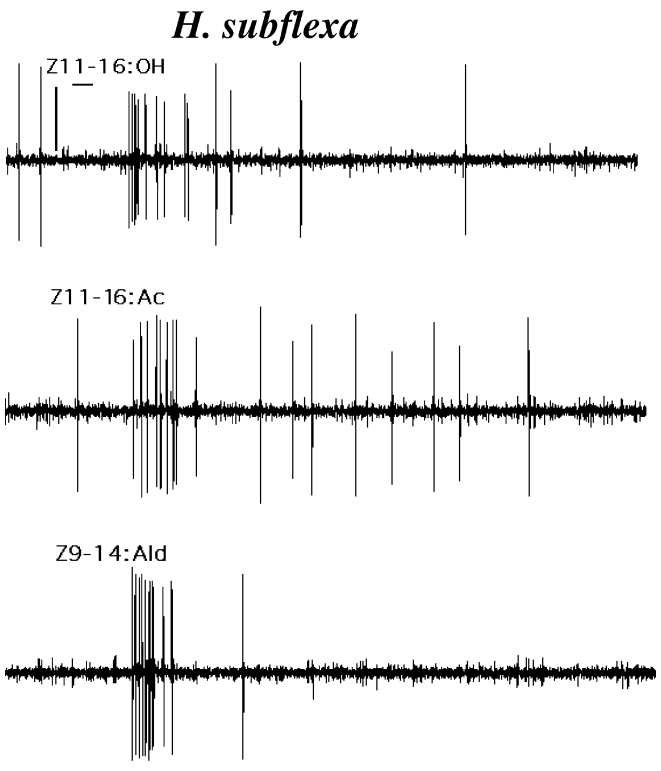


Fig. 3 Responses of ORNs in the C-type sensilla on *H. subflexa* male antenna showing that the action potential amplitudes of the ORN responding to Z11–16:OH are smaller than those of the ORN that responds to both Z11–16:Ac and Z9–14:Ald. *Horizontal bar* denotes stimulus delivery of 20 ms; *vertical bar* indicates 5 mV. Stimulus loadings were 3 μg Z11–16:OH, 3 μg Z11–16:Ac, and 10 μg Z9–14:Ald

Z11–16:Ac, and Z9–14:Ald (Fig. 2C). Analysis of the impulse amplitudes showed that two ORNs were involved; a smaller-spike-amplitude ORN responded to Z11–16:OH, and an ORN with a larger spike amplitude responded to both Z11–16:Ac and Z9–14:Ald (Figs. 2C, 3). Differential adaptation experiments (Fig. 4B) confirmed that Z11–16:OH stimulated the smaller-spiking ORN, and that Z11–16:Ac and Z9–14:Ald stimulated the same, larger-spiking ORN (Fig. 4B). A sample of the mean spike amplitudes from these sensilla showed that the mean amplitude of the ORN firing in response to Z11–16:OH was 11.75 ± 3.73 mV (mean $\pm$ SD; $n = 12$), whereas the amplitudes in response to Z11–16:Ac and Z9–14:Ald were 14.31 ± 3.86 mV and 14.68 ± 4.48 mV, respectively ($n = 12$).

In one out of the 24 sensilla of this type the spike-size/pheromone compound relationship was reversed: Z11–16:Ac and Z9–14:Ald both stimulated the smaller-spiking ORN and Z11–16:OH stimulated the larger spiking ORN. In addition, in approximately 25% of the cases, Z9–14:Ald caused both the large-spiking and the small-spiking ORNs to fire. Neither Z11–16:Ac nor Z11–16:OH was ever observed to stimulate both the large- and the small-spiking ORN. Dose-response profiles showed that the larger-spiking ORN exhibited approximately the same sensitivity to Z11–16:Ac as it did to

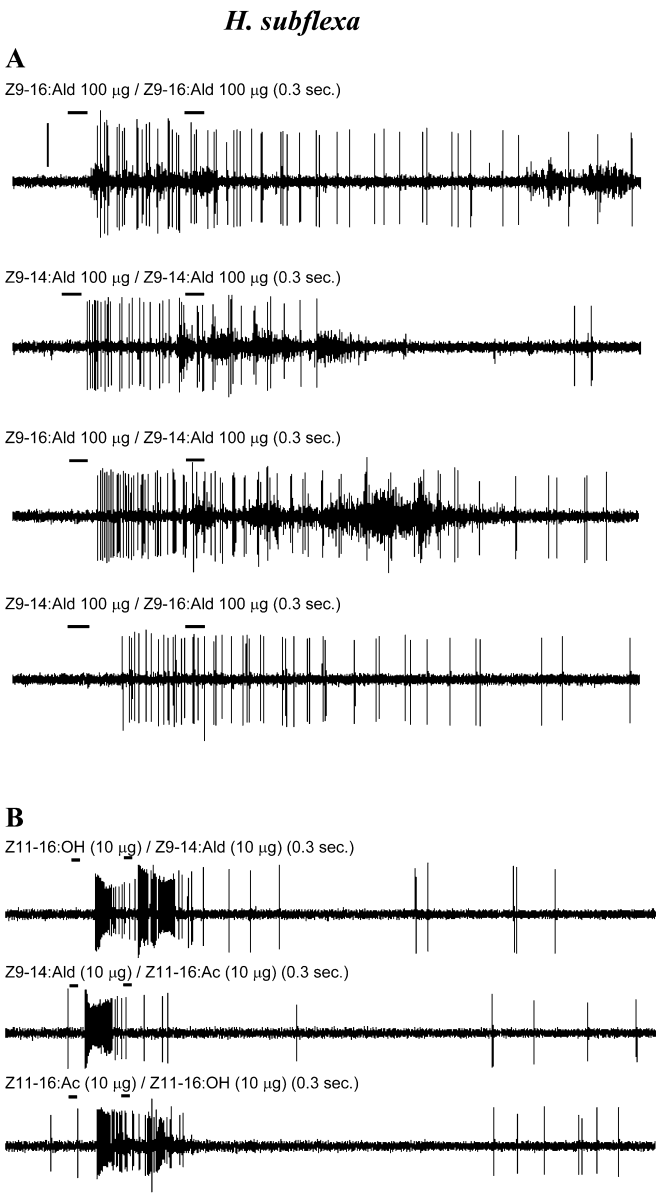


Fig. 4 A Spike-trains generated during cross-adaptation stimulation of an ORN in a *H. subflexa* B-type sensillum. Odorants that were puffed, and their sequence, are as indicated. *Small horizontal bars* represent 50-ms puffs through the odor cartridges. Regardless of whether Z9–16:Ald or Z9–14:Ald was the initial stimulus, no change in spike frequency (or amplitude) occurred following a second stimulation with either the same or different odorant. The results indicate that the same ORN is responding to both Z9–14:Ald and Z9–16:Ald. *Vertical bar* denotes 5 mV. **B** Spike-trains generated from *H. subflexa* ORNs within C-type sensilla during cross-adaptation stimulation trials. Odorants that were puffed, and their sequence, are indicated in the figure. *Small horizontal bars* represent when 50 ms puffs through the odor cartridges occurred. In the first spike record (*upper trace*), the spike activity of the smaller-spiking ORN caused by the initial puff of Z11–16:OH does not affect the response of the larger-spiking ORN stimulated 0.3 s later by Z9–14:Ald. In the second spike record (*middle trace*), this larger spiking ORN, stimulated initially by Z9–14:Ald, is adapted and unable to respond to a subsequent puff of Z11–16:Ac. In the third spike record (*bottom trace*), the activity of this larger-spiking ORN, stimulated initially by Z11–16:Ac, does not affect the subsequent response of the smaller-spiking ORN to Z11–16:OH. Same vertical scale as in **A**, denoted by 5-mV *vertical bar in uppermost trace* in **A**

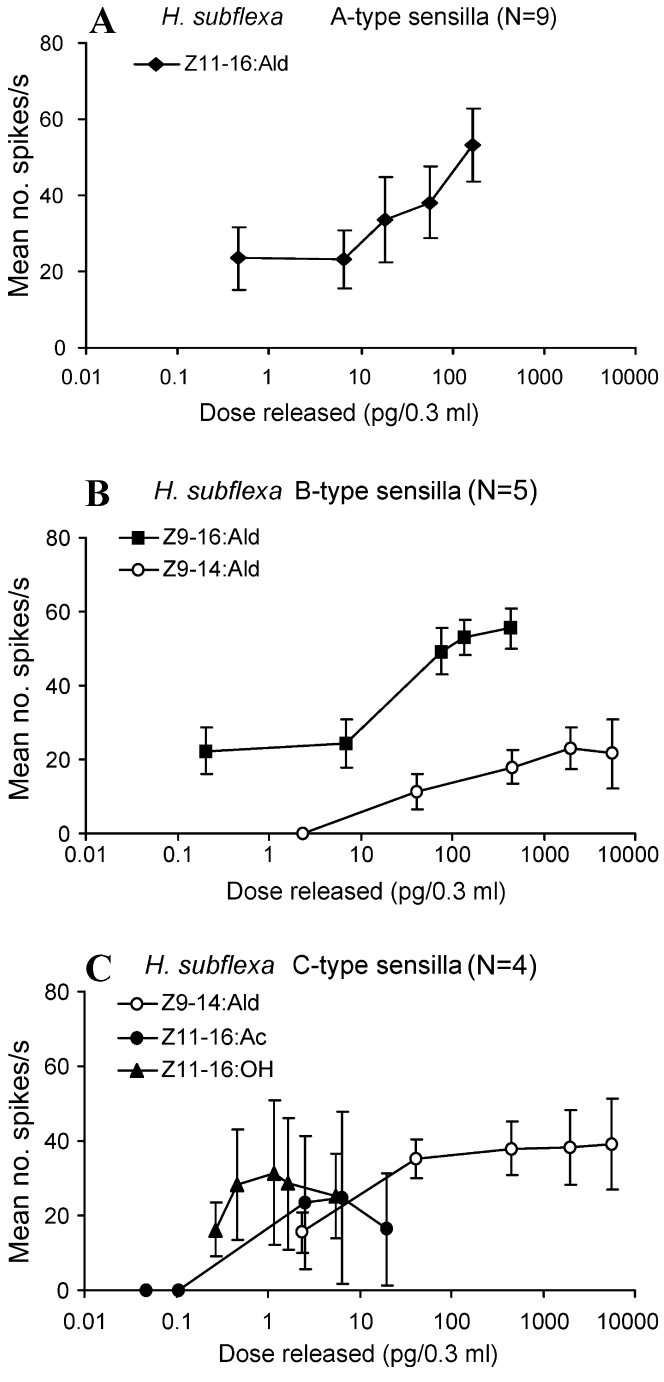


Fig. 5 Dose-response profiles of ORNs housed within **A** type A, **B** type B, and **C** type C sensilla on *H. subflexa* male antennae. These profiles are constructed using the actual amounts we measured as being emitted from odor cartridges containing filter papers loaded with either 3, 10, 30, 100, or 300 μg of the respective compounds during a 0.3-ml puff (Cossé et al. 1998). Spike frequency (spikes s^{-1}) was calculated by counting the spikes occurring during the first 0.2 s of response and dividing by 0.2. *n* is the number of sensilla sampled. Error bars denote standard errors of the mean (±SEM)

Z9–14:Ald (Fig. 5C). Likewise, the smaller-amplitude ORN exhibited approximately the same sensitivity to Z11–16:OH as did the larger amplitude ORN to Z11–16:Ac or Z9–14:Ald (Fig. 5C).

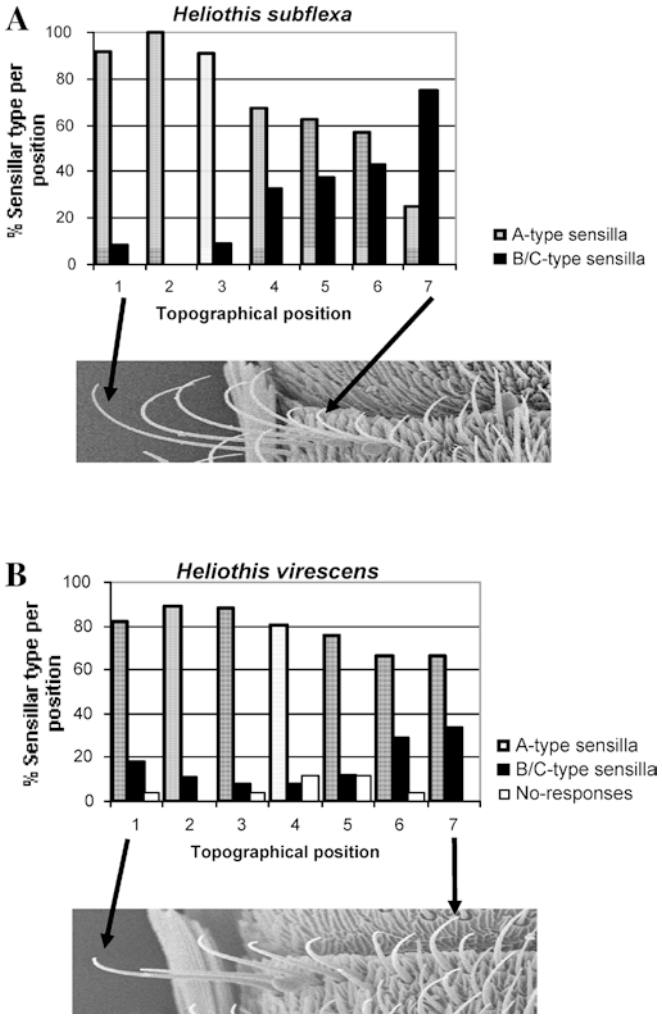


Fig. 6 **A** Topographical distribution of sensillar types on flagellomere no. 20 of *H. subflexa* antennae ($n = 196$ sensilla). Sensillum 1 (*far left*) is the longest, most equatorial of the sensilla trichodea and sensilla 2–7 become progressively smaller toward the most ventral, central region of the chemoreceptive antennal surface. For the number of sensilla numbers 1–7 that were sampled, $n = 37$, 33, 33, 34, 32, 21, and 16, respectively. **B** Topographical distribution of sensillar types on flagellomere no. 20 of *H. virescens* antennae ($n = 172$ sensilla). Sensillum 1 (*far left*) is the longest, most equatorial of the sensilla trichodea and sensilla 2–7 become progressively smaller toward the most ventral, central region of the chemoreceptive antennal surface. For the number of sensilla numbers 1–7 that were sampled, $n = 28$, 28, 26, 26, 25, 24, and 15, respectively

Finally, in ca. 2% of the sensilla sampled (4 out of 292) there were no responses from ORNs. In these cases, background spiking activity was recorded, but there were no responses to any of the odorants puffed over these sensilla at any dose.

The defined sample transects made across antennal flagellomere (pseudo segment) number 20 ($n = 196$ sensilla) showed that the A-type sensilla are found in greater abundance in the longest sensilla trichodea nearer the equatorial region of the segment (closer to the non-olfactory scales; Figs. 1A, 6A). The B- and C-type

sensilla, on the other hand, are found more commonly in the shorter sensilla trichodea that are located toward the middle (ventral) surface of the segment, farthest from the scales along either border (Figs. 1A, 6A).

H. virescens

We recorded from 403 sensilla using 100 male *H. virescens* antennae. As in *H. subflexa*, we found three distinctly different response profiles occurring in these recordings. ORNs in 81% of these sensilla (326 out of 403) responded only to the major pheromone component, Z11–16:Ald (Fig. 7A), and exhibited no activity to any of the other pheromone-related compounds.

ORNs in 3% of the sensilla (14 out of 403) responded only to the *H. virescens* secondary pheromone component Z9–14:Ald (Fig. 7B). Dose-response curves indicated that the ORNs in this type of sensillum responded to the lowest emission (2 pg) of Z9–14:Ald that issued from the odor cartridge in a 50-ms puff (Fig. 9B).

In the third type of sensillum encountered, comprising 16% of those sampled (63 out of 403), ORN responses were readily evoked by puffs of both Z11–16:OH and Z11–16:Ac, with occasional responses to very high emission rates of Z9–14:Ald (Fig. 7C). In some of the recordings (cf. Fig. 8A), distinctly different impulse amplitudes could be discerned in the responses to Z11–16:OH (smaller spikes) compared with those to Z11–16:Ac (larger spikes). Cross-adaptation studies ($n = 21$ sensilla) confirmed the presence of two ORNs usually having different spike sizes (Fig. 8B), and also showed that even when no differences in impulse amplitude could be discerned (in ca. 30% of the sensilla sampled), there were two different ORNs responding to Z11–16:Ac and Z11–16:OH, respectively. In one recording out of 21, the spike-size relationships were reversed, with the smaller-spiking ORN responding to Z11–16:Ac and the larger-spiking ORN responding to Z11–16:OH. Dose-response profiles showed that both of these ORNs are extremely sensitive to the compounds to which they are tuned, with responses starting at puffs of less than ca. 1 pg of either Z11–16:OH or Z11–16:Ac (Fig. 9C). However, responses to Z9–14:Ald by the smaller-spiking ORN occurred at excessively high emitted amounts, over 1,000 times the amount causing responses to either of the other two compounds (Fig. 9C).

Defined sample transects ($n = 172$ sensilla) made across antennal flagellomere number 20 showed that A-type sensilla on *H. virescens* male antenna tend to be slightly more common in the longest sensilla trichodea nearer the equatorial region of the segment (closer to the non-olfactory scales; Fig. 6B). The B- and C-type sensilla, on the other hand, are found slightly more commonly in the shorter sensilla trichodea that are located toward the middle (ventral) surface of the segment, farthest from the scales (Fig. 6B). This trend was not as pronounced, however, as it was in *H. subflexa* males. In ca. 5% of the sensilla sampled during the transect sampling (9 of the 172) there were no responses from ORNs.

Discussion

The organization of sex pheromone-component-sensitive ORNs within the sensilla trichodea on male *H. subflexa* and *H. virescens* antennae is similar to that of a third North American heliothine species, *H. zea* (Cossé et al. 1998) (Fig. 10). In all three species, the majority (70–80%) of sensilla are of type A, containing only one pheromone-component-responsive ORN that responds to the major sex pheromone component Z11–16:Ald.

Similarly, a second type of sensillum comprising 3–18% of the sensilla trichodea sampled in all three species contains an ORN responsive to Z9–14:Ald (Fig. 10). Notably, the ORN in these type B sensilla responds to Z9–16:Ald only in *H. subflexa* (Figs. 2B; 4, 10). Our dose-response results indicate that this ORN is actually more sensitive to Z9–16:Ald, the *H. subflexa* secondary pheromone component (Vickers 2002), than to Z9–14:Ald (Fig. 5B). Based on the Z9–14:Ald dose-response profile of this ORN (Fig. 5B), one could predict that in *H. subflexa* Z9–14:Ald might be able to substitute behaviorally for Z9–16:Ald if it were present in a blend with Z11–16:Ald plus the other important secondary component, Z11–16:OH (Vickers 2002), but at perhaps 150 times the aerial concentration of the Z9–16:Ald it is replacing. Of course, it is possible that the concomitant activity of the ORN in the C-type sensillum that also responds to Z9–14:Ald might somehow interfere with any behavior resulting from this substitution.

A third type of sensillum trichodeum in all three species, the C-type, comprises ca. 10–20% of the hairs sampled (Fig. 10). In the present study, we found that in the *H. subflexa* C-type sensillum there are two ORNs, one that is tuned to the essential secondary pheromone component Z11–16:OH (Vickers 2002), and another that is tuned to the possible (but only weakly behaviorally active) secondary pheromone component, Z11–16:Ac (Vickers 2002) (Fig. 10). The Z11–16:Ac-sensitive ORN in *H. subflexa* is approximately as sensitive to Z9–14:Ald as it is to Z11–16:Ac. However, despite numerous trials, there is as yet no known behavioral effect of Z9–14:Ald on *H. subflexa* males when it is added to the blend of Z11–16:Ald, Z11–16:OH, and Z9–16:Ald, or to partial blends lacking one of the secondary components (Vickers 2002).

Our study also confirms and extends the results of Berg et al. (1998) that the sensillum that we are calling the 'C-type' in *H. virescens* contains not one, but two ORNs, and now also that each is equally highly sensitive to their ligands, either Z11–16:OH or Z11–16:Ac (Fig. 10). In our study, with but one exception the large-spiking ORN was tuned to Z11–16:Ac and the small-spiking ORN was tuned to Z11–16:OH, whereas Berg et al. (1998) reported the reverse spike size relationship in the two sensilla they recorded from in odorant test series that included Z11–16:OH. In other series lacking Z11–16:OH but instead including Z9–14:Ald (series that Berg et al. employed in order to evoke spiking from what they thought was a Z9–14:Ald-tuned ORN but

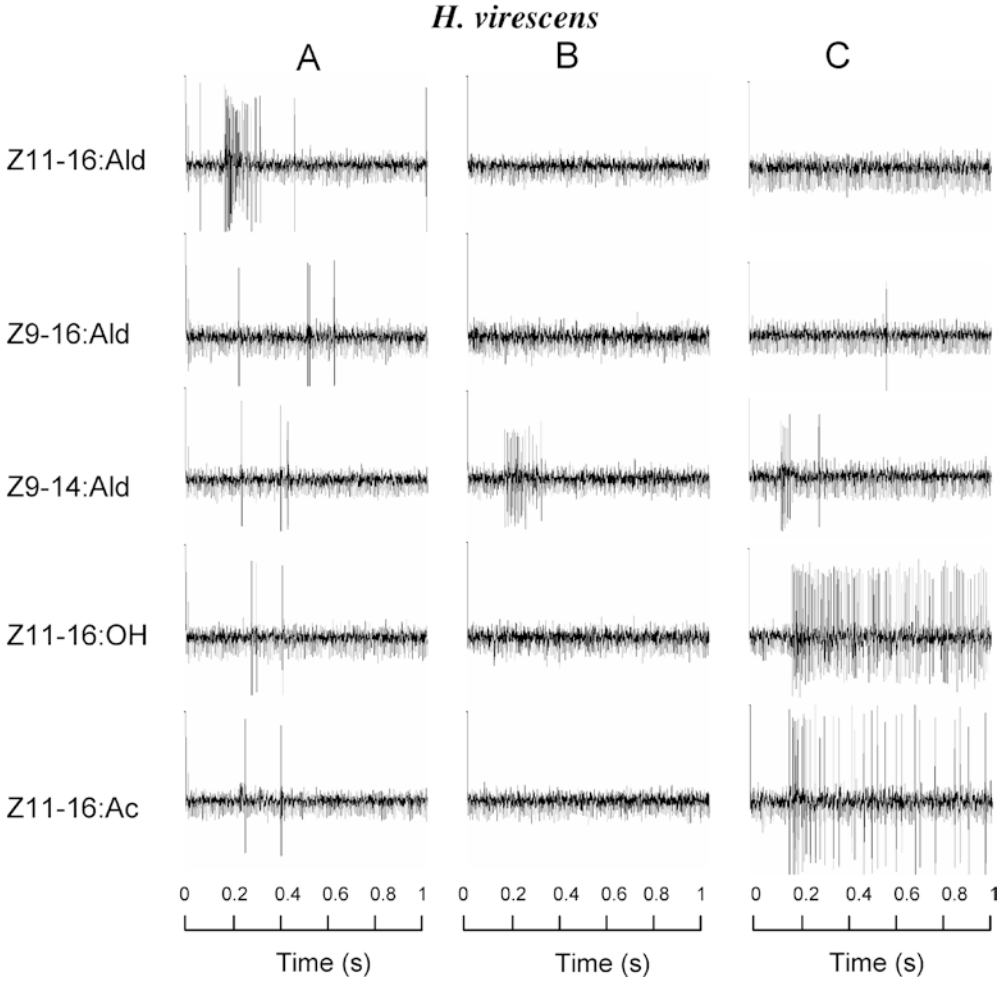


Fig. 7 Spike-train response profiles of male *H. virescens* ORNs housed within three types of sensilla we have named type A (**A**), type B (**B**), and type C (**C**). Type A sensilla house a single odorant-responsive ORN that fires in response to Z11–16:Ald. In type B sensilla, the only spike trains were generated in response to Z9–14:Ald, with no responses to Z9–16:Ald. In type C sensilla, there is a larger-amplitude-spiking ORN that responds to Z11–16:Ac at low amounts, as well as a second, smaller-amplitude spiking ORN that responds to Z11–16:OH at low amounts. This latter ORN also responds to Z9–14:Ald, but only at extremely high emitted amounts. Responses to a blank odor cartridge (not shown) are similar in appearance to the responses to Z9–16:Ald by the ORNs in types A, B and C sensilla. Loadings in the cartridges used to evoke these spike trains were: 100 μg Z11–16:Ald, 100 μg Z9–16:Ald, 100 μg Z9–14:Ald, 100 μg Z11–16:OH, and 100 μg Z11–16:Ac, respectively

subsequently turned out to be the Z11–16:OH-tuned ORN), Berg et al. (1998) showed that the Z11–16:Ac-responsive ORN exhibited smaller spikes than the ORN responding to Z9–14:Ald. We do not know the reason for this disparity in spike size relationships, but nevertheless, the two studies are in agreement with regard to the co-compartmentalization of these two types of ORNs and the excitation of the Z11–16:OH-tuned ORN by Z9–14:Ald in this type of sensillum.

The arrangement of ORNs in *H. virescens* C-type sensilla differs from that of the ORNs in the *H. subflexa* C-type sensilla (Fig. 10). Although in our study in both species the larger-spiking ORN is nearly always the one that is tuned to Z11–16:Ac, in *H. subflexa* this ORN is also almost as sensitive to Z9–14:Ald as it is to Z11–16:Ac. In contrast, in *H. virescens* the Z11–16:Ac-responsive ORN does not respond at all to Z9–14:Ald. Moreover, it is the smaller-spiking Z11–16:OH-tuned ORN that responds to Z9–14:Ald, although we now consider it debatable whether this extremely low level of

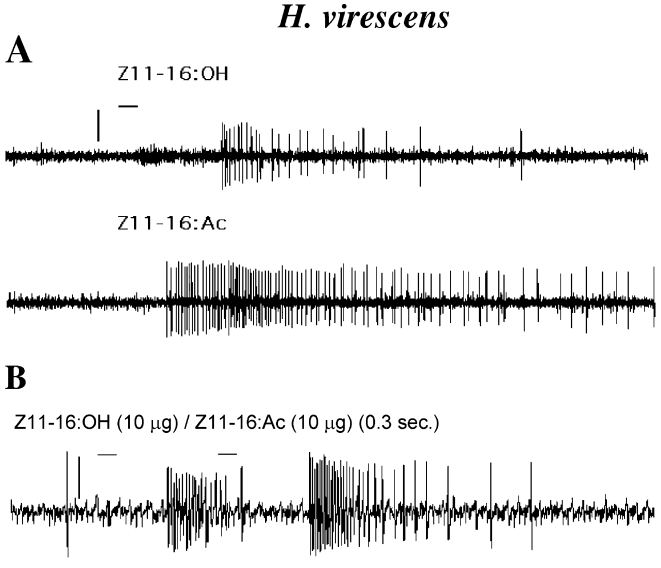


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Fig. 8 A Spike trains from a *H. virescens* type C sensillum following stimulation with Z11–16:OH (*top*) and with Z11–16:Ac (*bottom*) loaded at loadings of 100 μg in odor cartridges. Smaller spike amplitude of the ORN stimulated by Z11–16:OH is apparent. *Horizontal bar* indicates 50 ms; *vertical bar* denotes 5 mV. **B** From a different *H. virescens* type C sensillum, spike trains recorded during differential adaptation with a puff from a 10-μg Z11–16:OH cartridge (*left horizontal bar*) followed 0.3 s later by a puff of Z11–16:Ac (*right horizontal bar*), also from a 10-μg cartridge. *Horizontal bars* indicate 50 ms; *vertical bar* denotes 5 mV

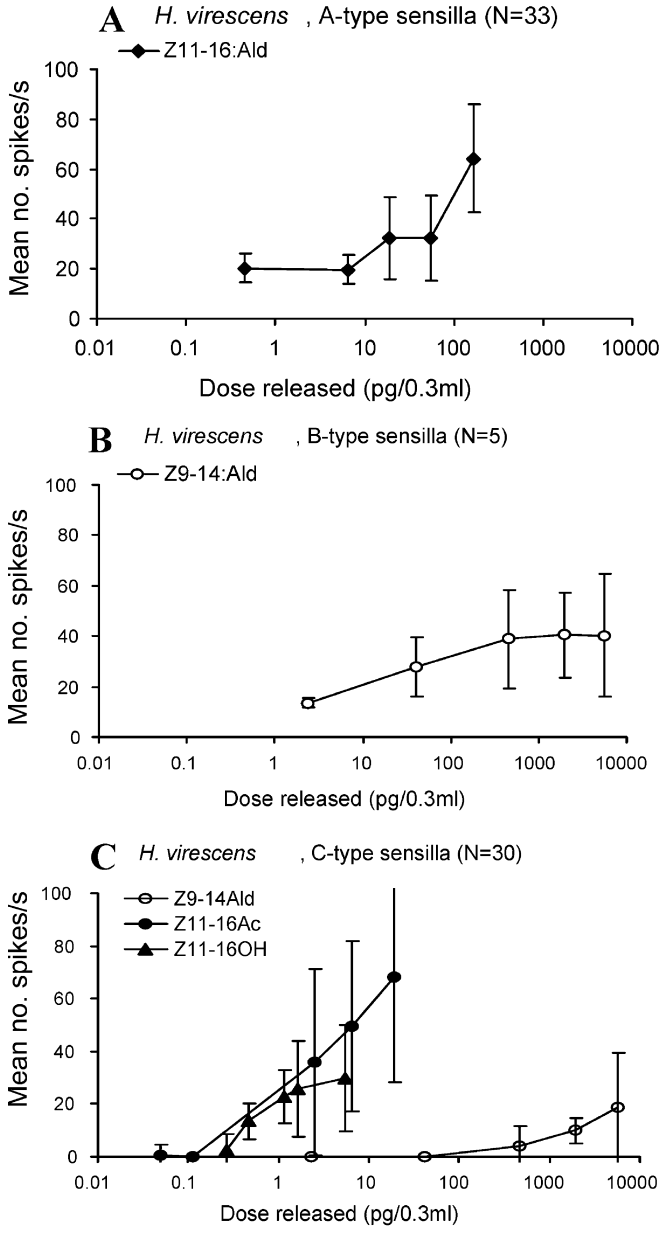

Fig. 9 Dose-response profiles of ORNs housed within **A** type A, **B** type B, and **C** type C sensilla on *H. virescens* male antennae. These profiles are constructed using the actual amounts we measured as being emitted from odor cartridges containing filter papers loaded with either 3, 10, 30, 100, or 300 μg of the respective compounds during a 0.3-ml puff (Cossé et al. 1998). Spike frequency (spikes s^{-1}) was calculated by counting the spikes occurring during the first 0.2 s of response and dividing by 0.2. *n* is the number of sensilla sampled. Error bars denote ±SEM. Dosages used in the odor cartridges to result in the emission rates shown for all stimuli were 3, 10, 30, 100, and 300 μg, respectively

responsiveness only to high emitted dosages of Z9–14:Ald is biologically significant.

In *H. subflexa*, the activity of the ORN in the C-type sensilla responding to Z11–16:OH contributes to pheromone-blend-related attraction, because Z11–16:OH is a critical secondary pheromone component; Z11–16:Ac appears to be behaviorally neutral when added to the blend of other components (Vickers 2002). Neither Z11–16:Ac nor Z9–14:Ald (that also occasionally simultaneously stimulates the Z11–16:OH-tuned ORN) are behaviorally antagonistic, even at high dosages (Vickers 2002).

In contrast, in *H. virescens*, both Z11–16:Ac and Z11–16:OH have been shown to act as behavioral antagonists when present in low proportions in the otherwise attractive *H. virescens* pheromone blend (Vetter and Baker 1983; Vickers and Baker 1997; Baker et al. 1998). Z11–16:Ac exerts antagonism when present at lower proportions in the blend (0.1–1.0%) (Vickers and Baker 1997) than does Z11–16:OH (>3.0%; Vetter and Baker 1983). Interestingly, in *H. virescens* field trapping as well as wind-tunnel tests, Z11–16:OH occasionally appeared capable of acting agonistically to increase attraction (Ramaswamy et al. 1985; Sparks et al. 1979; Hartstack et al. 1980) when added in small proportions to the sex pheromone blend. The presence of two separate ORNs tuned to these two different compounds would help explain the inconsistent, disparate behavioral effects of attraction and antagonism sometimes evoked by Z11–16:OH and the consistent antagonism evoked by Z11–16:Ac (Vickers and Baker 1997).

The dose-response profiles of ORNs sampled in the C-type sensilla provide definitive characters for discriminating between the male antennae of *H. subflexa* and *H. virescens*. In *H. subflexa*, it is the Z11–16:Ac-tuned ORN that responds to Z9–14:Ald with only about three times less sensitivity as to Z11–16:Ac. Also, Z9–14:Ald is capable of generating high spike frequencies in response to increasingly high doses of Z9–14:Ald. In contrast, in *H. virescens* it takes extremely high emitted doses of Z9–14:Ald (>1,000 times that of Z11–16:OH) to elicit any spiking activity at all, and it is the Z11–16:OH-tuned ORN that responds to Z9–14:Ald. Moreover, these spike frequencies remain low even at high emitted dosages of Z9–14:Ald.

In addition, the B-type sensilla provide a reliable way to distinguish between *H. virescens* and *H. subflexa* antennae, although B-type sensilla are difficult to find, especially in *H. virescens*. In *H. subflexa*, B-types contain one ORN that responds predominantly to Z9–16:Ald but also to Z9–14:Ald at markedly higher emitted dosages. In contrast, in *H. virescens* the single pheromone-component-sensitive ORN housed in the B-type sensilla responds only to Z9–14:Ald.

The finding of only one pheromone-compound-sensitive ORN in A-type sensilla in *H. subflexa, H. virescens* (and *H. zea*, Fig. 10) does not mean that there is only one ORN housed in these sensilla. Cobalt stainings that we have recently performed within A-type sensilla from *H. zea* have routinely shown two ORNs that stain and project to different regions of the antennal lobe (S.G. Lee, S.A. Ochieng, and T.C. Baker, unpublished observations). Similarly, Hansson et al. (1995), Almaas et al. (1991), and Berg et al. (1998) also found during attempts to stain ORNs responsive to Z11–16:Ald in *H. virescens* (from the same types of sensilla we have labeled 'A-type')

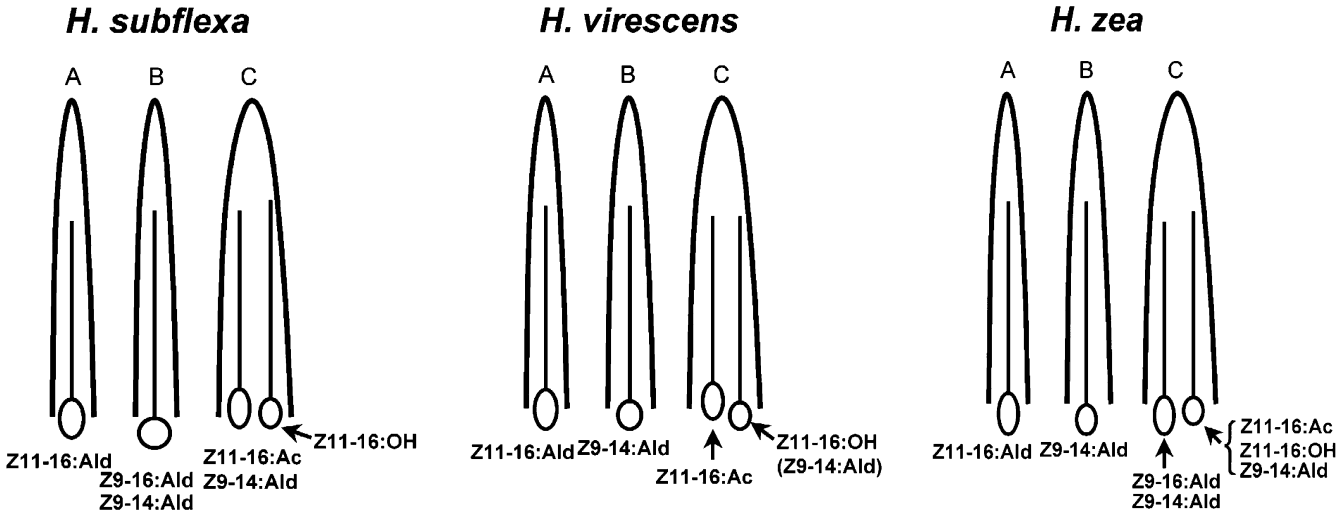

Fig. 10 Schematic comparison of results from sampling the ORNs in the types A, B, and C sensilla from *H. subflexa* and *H. virescens*. For comparison, we have included a depiction of the arrangement of ORNs in the sensilla of *H. zea* (Cossé et al. 1998). *Ovals* at the bases of vertical lines indicate cell bodies of the ORNs and the *lines* indicate dendrites housed within the conical depictions of the sensilla. *Larger ovals* represent cell bodies of larger-spiking ORNs than those depicted with smaller ovals. The compounds to which the various ORNs respond are indicated

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that a second ORN routinely was stained. Berg et al. (1998) discerned that in the most sensitive ORNs of these sensillar-types, there was a very small-spiking ORN that fired along with the large-spiking ORN when Z11–16Ald was used as the stimulus. During cobalt staining of such preparations, Berg et al. (1998) found that a second ORN that projected to an 'ordinary' medial and dorsal glomerulus was sometimes stained in addition to the Z11–16:Ald-sensitive ORN projecting to the cumulus (main glomerulus of the MGC, Vickers et al. 1998). Hansson et al. (1995) also reported a double staining in one of their attempts that occurred within a sensillum housing a Z11–16:Ald-sensitive ORN. The second stained ORN projected to a more dorsal and medial glomerulus (which they labeled 'b') relative to the cumulus (which they labeled 'a'), which always received projections when Z11–16:Ald-sensitive ORNs stainings were attempted. Berg et al. (1998) interpreted the 'b' glomerulus (Hansson et al. 1995) as being located outside the MGC, and as being perhaps the same ordinary glomerulus that received input from the silent ORN in their study.

Similarly, we have found that in *H. subflexa* that there is a second 'silent' ORN housed in A-type sensilla that takes up stain and shows an axonal projection destination to the dorsal-medial region of the antennal lobe in addition to the Z11–16:Ald-sensitive ORN projecting to the cumulus of the MGC (S.G. Lee, S. Ochieng, N.J. Vickers and T.C. Baker, unpublished observations). It remains to be seen whether these ORNs we consider to be 'silent' are in fact unresponsive to all odorants, or whether they are tuned to compounds we have not yet tested on them.

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