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Response profile of pheromone receptor neurons in male *Grapholita molesta* (Lepidoptera: Tortricidae)

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ABSTRACT

The response profile of olfactory receptor neurons (ORNs) of male *Grapholita molesta* (Busck) to the three female sex pheromone components [(*Z*)-8-dodecenyl acetate (*Z*8-12:Ac), (*E*)-8-dodecenyl acetate (*E*8-12:Ac), and (Z)-8-dodecenyl alcohol (*Z*8-12:OH)] was tested with single sensillum electrophysiology. Sensilla trichodea housed normally one, but sometimes two or three ORNs with distinct action potential amplitudes. One third of the sensilla contacted contained ORNs that were unresponsive to any of the pheromone components tested. The remaining sensilla contained one ORN that responded either to the major pheromone component, *Z*8-12:Ac ("Z-cells", 63.7% of sensilla), or to its isomer *E*8-12:Ac ("E-cells", 7.4% of sensilla). 31% of Z- and E-sensilla had 1 or 2 additional cells, but these did not respond to pheromone. None of the 176 sensilla contacted hosted ORNs that responded to *Z*8-12:OH. The proportion of Z- and E-cells on the antennae (100:11.6, respectively) is similar to the proportion of these compounds in the blend (100:6, respectively). The response of Z-cells was very specific, whereas E-cells also responded to the Z isomer, albeit with lower sensitivity.

1. Introduction

Grapholita molesta (Busck) larvae bore on new growth shoots of peach trees (*Prunus* spp.) reducing fruit yield (Rothschild and Vickers, 1991). The sex pheromone has been described as a 100:6:10 blend of (*Z*)-8 dodecenyl acetate (*Z*8-12:Ac), (*E*)-8 dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8 dodecenyl alcohol (*Z*8-12:OH), respectively (Roelofs et al., 1969; Beroza et al., 1973; Cardé et al., 1975a, 1979; Baker and Cardé, 1979; Baker et al., 1981; Linn and Roelofs, 1983), and is used for monitoring and mating disruption over 50,000 hectares of peach and apple around the world (Witzgall et al., 2010).

The behavioral response of *G. molesta* to pheromone and plant odours has been studied in detail (e.g., Linn and Roelofs, 1981; Linn et al., 1988, 1991; Willis and Baker, 1994, 1988; Piñero and Dorn, 2007, 2009; Ílichev et al., 2009; Varela et al., 2011a; Lu et al., 2012, 2013; Najar-Rodriguez et al., 2012, 2013; Trimble, 2012). Electroantennography has been used to explore questions mainly related to mating disruption (e.g., Stelinski et al., 2006; Molinari et al., 2010; Trimble and Marshall, 2010; Khuns et al., 2012; D'Errico et al., 2013; Faraone et al., 2013), and at the CNS level, the three-dimensional structure of the antennal lobe (AL), and the physiological response of AL neurons to pheromone and plant odours have been studied (Najar-Rodriguez et al., 2010; Varela et al., 2009, 2011b). In addition Nagy and George (1981) and George and Nagy (1984) described the neuroanatomy of sensilla and olfactory receptor neurons (ORNs) in males, and Baker et al. (1988) analyzed the effect of temperature on the ability of olfactory receptor neurons to detect pheromone pulses. However, a detailed characterization of the physiological response of pheromone receptor neuron types in *G. molesta* is lacking.

Pheromone ORNs make a large percentage of the receptor neurons on the male moth antenna and are extremely sensitive to low doses of sex pheromone (Kaissling, 2004). Electrophysiological studies in moths show specific pheromone component detection by distinct ORNs, which may be housed singly in a sensillum trichodeum or together with other pheromone ORNs (reviewed by De Bruyne and Baker, 2008; Baker et al., 2012). In general, there is a correlation between the proportion of ORNs that respond to the major and minor pheromone components and the relative abundance of these compounds in the female-produced sex pheromone blend (Baker et al., 2012).

The aim of our study was to characterize the physiological response of male *G. molesta* ORNs to the three components of the sex pheromone blend. We mapped the position of different sensillum types on the antennae by SEM, and recorded the response of

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ORNs housed in sensilla trichodea to several doses of the pheromone components. We expected that males of *G. molesta* would have ORNs specific for each of the three pheromone components, and that these would be present in a proportion similar to the proportion of the pheromone components in the pheromone blend. Similarly, we expected that these ORNs would be highly sensitive and specific to their respective ligands.

2. Materials and methods

2.1. Insects

The *G. molesta* colony originated from a laboratory rearing established at Piacenza, Italy, from insects collected in peach orchards in that locality, and was maintained at the University of Lleida, Spain, since 2005. Larvae were reared on a semi-synthetic diet modified from Ivaldi-Sender (1974) under a L16:D8 photoperiod at 25 ± 1 °C. Pupae were separated by sex and were placed in 4-L polypropylene containers provided with a cotton ball soaked in 10% sugar dissolved in water. Adults were separated daily and used when 2–4 days old. Care was taken not to expose adults to synthetic odor sources before the studies.

2.2. Scanning electron microscopy (SEM)

Male antennae were excised from the head with fine forceps. Scales were removed individually by hand under the stereomicroscope using a sharpened tungsten electrode, watching not to damage the sensilla hidden underneath. Antennae were mounted on SEM stubs lined with conductive double-side adhesive black tape, with the orientation of the mounted antenna to show the areas of interest. Preparations were air dried at room temperature for 3–4 days and then coated, using a sputter coater (Balzers SCD 050, Leica Microsystems, Madrid, Spain), with 50-nm gold particles for 3 min from a distance of 50 mm, with a current of 45 mA and Argon as cooling gas. Samples were examined in a scanning electron microscope (DSM 940A, Zeiss, Germany) at 10 kV and a working distance of 12 mm. The scale-free area of 9 antennae and the scaled area of 4 antennae, each from a different individual, were examined. Sensilla counts were made every 5th flagellomere, starting on the proximal one. Total sensilla count per antennae was estimated by extrapolating these counts to the other flagellomeres. The scale-free area, which covers one third of the perimeter of each flagellomere, was fully visible, but the scaled area, which covers the remaining of the flagellomere surface, was always partially obstructed from vision. Using characteristic landmark structures that indicated the sagittal axis on the scaled area we could extrapolate sensilla counts from the visible section of the scaled area to the section hidden from view. Abundance and pattern of distribution of all types of sensilla are reported. Length, and basal and tip widths of all types of sensilla ($N = 20$ sensilla from four different antennae) were measured.

2.3. Odourant stimuli

The pheromone compounds of *G. molesta*, (*Z*)-8-dodecenyl acetate (*Z*8-12:Ac), (*E*)-8-dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8-dodecenyl alcohol (*Z*8-12:OH) were provided by Pherobank (The Netherlands) with an initial purity ⩾99%. Gas chromatographic analysis revealed that *Z*8-12:Ac contained 0.38% *E*8-12:Ac, and that *E*8-12:Ac contained 0.24% *Z*8-12:Ac. Undiluted compounds were weighted and diluted in *n*-hexane to make 100 μg/μl stock dilutions. Serial 10-fold dilutions of the stock dilutions in *n*-hexane were prepared from the stock solution as needed.

2.4. Electrophysiological recordings

Males were immobilized with industrial grade CO_2 for 10 s, and were mounted on a handcrafted poly(methyl methacrylate) insect holder. The body was inserted through a hole drilled in the holder and the protruding head was restrained by fixing a piece of adhesive cloth tape between the head and the holder. The antennae were carefully laid on a slant surface lined with double sided sticky tape, and were oriented for easy access with the electrodes. To record from sensilla located on the scaled area, scales were removed by gently rolling the antennae on the sticky tape. Remaining scales were removed individually with the help of a tungsten electrode. Sub-millimetric smoking paper strips placed over the antennae and glued to the sticky surface prevented antennal torsion. A stereo microscope (objective 2×, oculars 25×, zoom range 0.8–12.5, Leica Microsystems, Madrid, Spain) was used to help in these operations and to visualize the recordings. These were obtained by means of electrolytically (20% KNO_2) sharpened tungsten microelectrodes (0.125-mm diameter, 99.98% purity, Advent Research Materials Ltd, England). The reference electrode was inserted in the head through the mouth parts. For electroantennogram recordings (EAG) the tip of the recording electrode was inserted in one of the most distal segments of one antenna. For single sensillum recordings (SSR) the recording electrode was situated near the base of a randomly chosen sensillum trichodeum and pushed gently inward with the help of a manual micromanipulator (NMN-25, Narishige, Japan) until action potentials (AP) were detected. Flagellomeres 10–35 were sampled. The signal from the recording electrode was pre-amplified (10× gain, Universal Single Ended Probe, Syntech, Germany), filtered, and digitized (IDAC-4, Syntech, Germany), and recorded and analyzed in a PC (AutoSpike v.3.9, Syntech, Germany). The setup was mounted on an anti-vibration table (63–511, TMC Ametek, USA) and was shielded by a Faraday case to reduce low frequency noise.

2.5. Odor stimulation

Dilutions were applied as 1 μl aliquots (1 μl micropipettes, Drummond Scientific Co., USA) on 1 × 20 mm *n*-hexane-pre-cleaned filter paper strips (#1, Whatman International Ltd, England). After having dried (5 min) the filter papers were introduced in *n*-hexane-pre-cleaned 100 μl glass micropipettes (1.2 mm internal diameter, Blaubrand® Intramark, Germany) which were then stored in glass test tubes sealed with PTFE-coated screw caps until used. New stimuli cartridges were prepared each day, and a given cartridge was not used for more than 10 stimulations. Air flow was generated by two diaphragm aquarium pumps connected to a 3-way solenoid valve (CS-55, Syntech, Germany). A 0.5 l/min flow of charcoal-filtered and humidified air blew continuously over the insect preparation through a 5-mm internal diameter plastic tube placed 15–20 mm from the preparation (air velocity at exit = 0.4 m/s). The tip of the odor cartridge bearing the filter paper was positioned 0.4 cm down from the recording point and perpendicular to the direction of the continuous air flow. A 0.2 l/m charcoal-filtered room air flow was puffed through the odor cartridge to the recording area for 200 ms (air velocity at exit = 2.9 m/s). The flow of continuous humid air was decreased by 0.2 l/min during the puff. Time interval between puffs was at least 60 s, but longer if needed to let the spike activity return to pre-stimulation levels. A maximum of 5 cells were recorded per insect, and at least 30 min between two cell recordings were allowed. The air around the preparation was constantly renewed with an exhaust to minimize contamination. Test tubes were rinsed with acetone and heated at 250 °C overnight before reused.

2.6. Dose–response

Preliminary tests determined the range of concentrations to be used in the dose–response tests. For EAG we used 10, 100 and 1000 ng of each pheromone compound. For SSR the ORNs were first challenged with a high dose of each pheromone component (100 pg *Z*8-12:Ac, 1 ng *E*8-12:Ac, and 1 ng *Z*8-12:OH) to determine their physiological type (cells were typically more sensitive to one pheromone component and less sensitive to the others, see Section 3), and then dose–response curves were established in ORNs with stable contacts and good signal to noise ratio. The order of stimuli was first the negative control (*n*-hexane), followed by low to high doses of the test compounds. For each cell the full range of concentrations was tested for the most sensitive compound, whereas the range of concentrations tested for the other two compounds depended on the cell type. "Z-cells" were very sensitive and specific to *Z*8-12:Ac and were challenged with the full range of concentrations of *Z*8-12:Ac, but only with the two highest dosages of the other two compounds (except for a subset of 4–16 cells that were tested with the full range of *E*8-12:Ac). "E-cells" were most sensitive to *E*8-12:Ac, and moderately sensitive to *Z*8-12:Ac, so they were tested with the full concentration range of the two acetate isomers, but only with the two highest concentrations of the alcohol. Non-linear regression functions were fitted to the observed data (Byers, 2013). To correct for the 0.38% of *E*8-12:Ac present in *Z*8-12:Ac, the regression function of E-cells stimulated with *E*8-12:Ac was used to estimate how much of the response of the E-cells to stimulation with *Z*8-12:Ac was due to the *E*8-12:Ac contaminant, and the estimated response to the contaminant was subtracted from the observed response to *Z*8-12:Ac. Non-linear regression parameters were calculated using self-starting functions in R software (Crawley, 2009; R Core Team, 2012).

2.7. Cross-adaptation

A cross-adaptation test was performed to determine if the dual response of E-cells to *E*8-12:Ac and *Z*8-12:Ac was the result of (a) one ORN responding to both compounds, or (b) two ORNs of equal spike amplitude sharing the same sensillum but each responding to one of the two isomers. Two pheromone cartridges were angled at 45 degrees of each other with the outlets pointing to the SSR preparation. Each cartridge was connected to a different air flow and solenoid valve so that they could puff independently with the same airflow conditions described above. Once contact was established with an ORN it was first stimulated with *Z*8-12:Ac and *E*8-12:Ac to determine its type (Z- or E-cell, see Section 3). For cross-adaptation a single 200-ms puff from the first cartridge was followed by a 100-ms inter-stimulus interval and then by a 200-ms puff from the second cartridge. All possible combinations of the E- and Z-isomers (E and E, Z and E, E and Z, Z and Z) were tested in a given cell. The position (left or right pipette) of the stimulus was randomized among replicates. *Z*8-12:Ac and *E*8-12:Ac were puffed at 100 pg and 1 ng, respectively, in Z-ORNs, and at 1 ng and 100 pg, respectively, in E-ORNs. These concentrations were chosen based on the dose–response curves.

2.8. Spike analysis

When more than one spike size was detected they were sorted by amplitude. For each puff the number of spikes during a 1-s pre-stimulation period was subtracted from the number of spikes during a 1-s post-stimulation period. Peri-stimulus time histograms (PSTH) were plotted by grouping spikes in 25-ms bins starting at the onset of stimulation.

The response to *Z*8-12:Ac was more tonic in Z-cells than in E-cells. To determine if this difference was significant we calculated the times of half-rise and half-fall PSTH peak response, relative to the spontaneous activity of the cell (averaged for 1 s pre-stimulation). PSTHs of Z cells and E-cells to 100 pg and 1 ng loads of *Z*8-12:Ac, respectively, were normalized relative to the peak response, and 2nd order polynomial equations were fitted to the rise and fall phases. Estimated half-rise and half-fall times of cells stimulated with *Z*8-12:Ac were compared between Z- and E-cells with *t*-tests.

3. Results

3.1. Morphology

The antenna of *G. molesta* males is filiform and consists of 2 basal segments, scape and pedicel, and a flagellum composed of 45 flagellomeres, with no variation in number of flagellomeres among the 13 antennae from 13 different males analyzed. The flagellum carries most of the sensilla on the antenna. The dorsal and lateral areas of the flagellum bear scales, while a ventral band running the entire length of the flagellum (about 30% of the flagellomeres surface) remains scale-free. The apical flagellomere does not bear any scales.

Scanning electron microscopy of antennae revealed 6 different types of sensilla on the flagellomeres: trichodea, chaetica, coeloconia, auricillica, basiconica and styloconica (Fig. 1 and Fig. S1). The different types varied in distribution and density along the flagellum and between scaled and scale-free areas (Fig. S2). Sensilla trichodea were thin and long, (Table S1) and were surrounded by a socket-like structure. There were 2291 sensilla trichodea per antennae (1015.56 ± 78.71 and 1276 ± 42.84, mean ± SEM, in scaled and scale-free areas, respectively), which constituted 72% of all the sensilla (Table S2). Their number increased steeply between flagellomeres 1 and 5, remained high between flagellomeres 5 and 35, and decreased steeply from flagellomere 35 towards the distal end of the antenna (Fig. S2). The average number of sensilla trichodea per flagellomere in flagellomeres 5–35 was similar in the scaled and scale-free areas (mean ± SEM, 34.9 ± 0.4 and 34.9 ± 0.6, respectively), and therefore sensilla trichodea were denser in the smaller scale-free area than in the twice larger scaled area (Table S2, Fig. 1). Spatial distribution of sensilla trichodea in a flagellomere was random in the scale-free area, and arranged in rows in between the scales in the scaled area (Fig. 1). The second most abundant type of sensilla on the antennae of *G. molesta* males was sensilla auricillica (Table S2, Fig. 1 and Fig. S2). These are much shorter than sensilla trichodea and flattened rather than cylindrical, with a more variable range of sizes and shapes than sensilla trichodea (Table S1, Fig. 1 and Fig. S1). Sensilla auricillica constituted 11% of the total sensilla in the antenna and were more abundant in the scaled area than in the scale-free area (Table S2, Fig. 1 and Fig. S2). They always occurred on the distal area of the flagellomere, being more numerous on the lateral sides of the scaled area, and lacking in the central section of the scaled area (Fig. 1). The third most abundant type of sensilla (7% of the total sensilla) were the coeloconica (Table S2). These are easy to recognize by the central dome surrounded by 12–13 finger-like projections (microtrichia) (Fig. 1 and Fig. S1). Their distribution overlapped with sensilla auricillica. Sensilla basiconica made only 4% of the total sensilla. They look similar to sensilla trichodea but are comparatively shorter and wider (Table S2 and Fig. S1). They are present both in scaled and scale-free areas of the antennae.

The remaining two types of sensilla, chaetica and styloconica, were present in constant number and position in each flagellomere and served as topographic landmarks (Fig. 1). Each flagellomere, except the apical, bears one sensillum styloconica at the distal end of the mid-ventral area (Fig. 1). It consists of a finger-like

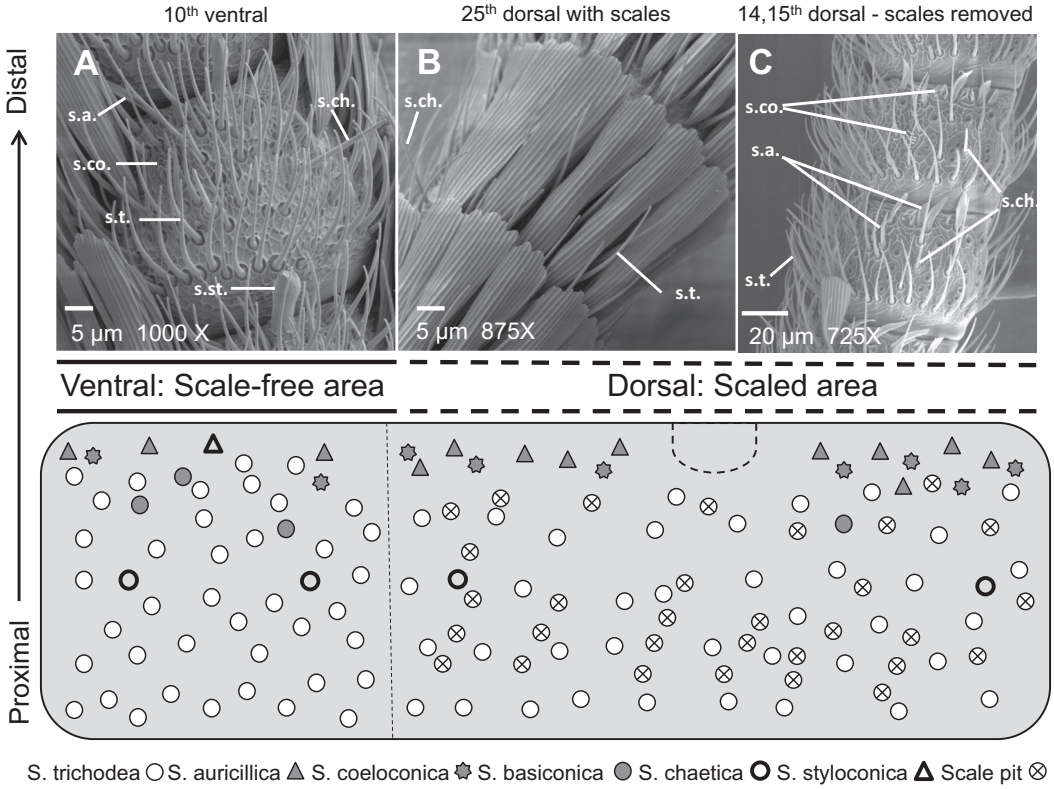

Fig. 1. Distribution of sensilla types on the flagellum of *G. molesta* males. The top part of the graph shows SEM pictures of (A) the scale-free ventral region, (B) the dorsal region, which is covered with scales, and (C) the dorsal region when the scales have been removed. All sensillum types are seen on the scale-free ventral region (except the basiconica which are not visible in this SEM figure), whereas the scale-bearing dorsal region shows only sensilla trichodea and chaetica (B). All other sensillum types (except styloconica, and the basiconica in this SEM picture) are visible in the scaled area when the scales are removed (C). The bottom part of the graph shows a schematic representation of the number and position of the different sensilla types for a prototypical flagellomere. The dashed line in the scaled area indicates a protuberance on that area of the flagellomere. Sensilla auricillica (s.a), sensilla basiconica (s.b), sensilla chaetica (s.ch), sensilla coeloconica (s.co), sensilla styloconica (s.st.), and sensilla trichodea (s.t).

structure, with a large pore at the terminal end (Fig. S1). Sensilla chaetica are similar to sensilla trichodea but they can be distinguished from the former ones because sensilla chaetica have a bulbous socket at the point of insertion on the antenna and they are more electron-dense and perpendicular to the surface of the antennae than the trichodea. There are four sensilla chaetica in the equator of each flagellomere, two on the laterals of the scaled area, and two on the scale free area (Fig. 1).

3.2. Specificity and sensitivity of pheromone ORNs

3.2.1. ORN types

Sensilla trichodea can be categorized in three distinct groups based on the response of their ORNs to pheromone stimuli. One group of sensilla housed a cell that was very sensitive to the major pheromone component, *Z*8-12:Ac, and responded very little to the highest concentrations of *E*8-12:Ac and *Z*8-12:OH (Fig. 2A). These were called Z-sensilla and Z-cells. A second type of sensilla contained a cell that was most sensitive to *E*8-12:Ac, showed intermediate response to Z8-12:Ac, and responded very little to the highest concentrations of *Z*8-12:OH (Fig. 2B). These were called E-sensilla and E-cells. Z- and E-sensilla made 63.6% and 7.4% of the sensilla trichodea on the antennae, respectively (Table S3). The rest of the sensilla (29%) contained cells that did not respond to any of the three pheromone components, as determined with a single high-concentration pheromone puff (Table S3). Out of 176 sensilla sampled for their response to the three pheromone components, we did not find a single ORN that responded to *Z*8-12:OH with similar sensitivity as the Z- and E-cells to their own ligands.

Z-sensilla were located along most of the length of the flagellomere (flagellomeres 10–35), whereas E-sensilla were usually located in the distal mid-dorsal (scaled) and proximal mid-ventral (scale-free) areas of the flagellomere. The proportion among sensilla types (Z, E and non-responding) was independent of flagellum area (scaled or scale-free) ($\chi^2 = 4.04$, df = 2, $P > 0.132$, Table S3). The proportion of Z-sensilla and sensilla with non-responding cells was significantly higher in the scale-free area than in the scaled area ($\chi^2 = 6.68$, df = 1, $P = 0.009$, and $\chi^2 = 7.44$, df = 1, $P = 0.006$, respectively), whereas E-sensilla were equally represented in both regions ($\chi^2 = 1.99$, df = 1, $P = 0.16$) (Table S3).

3.2.2. ORN number, spontaneous activity and spike amplitude

More than half (62.5%) of all the sensilla trichodea housed a single neuron of large spike amplitude (mean ± SEM, 1.81 ± 0.17 mV), whereas a smaller percentage (21.02%) housed two neurons, where one was of large amplitude, similar to that of the single neurons, and the second one of a smaller spike amplitude, and the remaining 16.48% sensilla housed 3 neurons (Tables S3 and S4). 84% of the Z-sensilla had a single neuron, whereas the percentage was lower in E-sensilla (54%) and still lower in sensilla with unresponsive cells (33%) (Table S3). The proportion of Z-sensilla with more than one ORN was significantly higher in the scale-free area than in the scaled area ($\chi^2 = 8.64$, df = 1, $P = 0.003$) (Table S3). In pheromone sensilla with more than one cell, only one of the ORNs responded to pheromone and in 84% of the cases it was the large amplitude neuron (Tables S3 and S4). The spike amplitude and spontaneous activity of Z- and E-cells was similar to each other and between single and paired cells (Table S4). Unresponsive cells were more

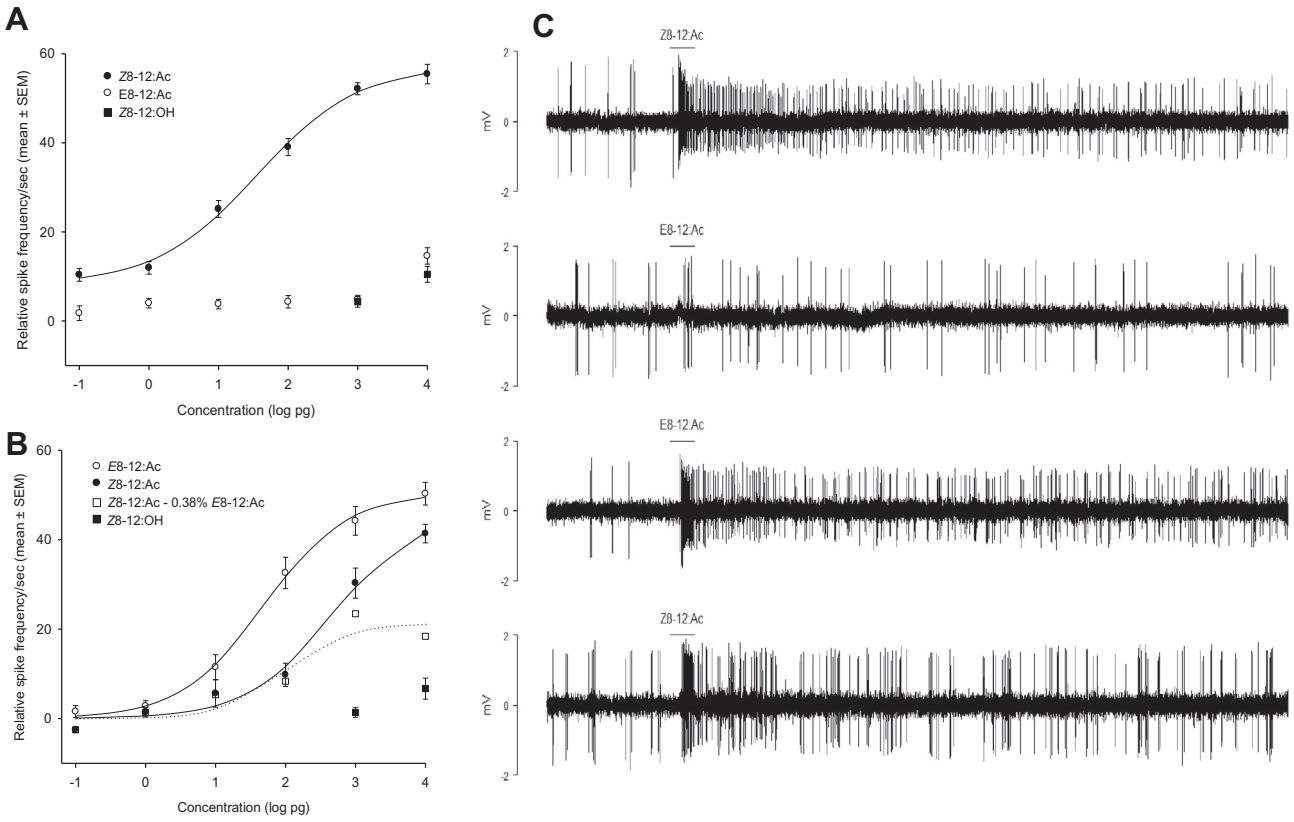

Fig. 2. Response of pheromone ORNs of *G. molesta* males to 200 ms puffs of each of the three pheromone components at several concentrations. Dots show observed data (mean ± SEM) and lines the adjusted curves. (A) *Z*8-12:Ac ORNs responded strongly to *Z*8-12:Ac and minimally to *E*8-12:Ac and *Z*8-12:OH. The response to *Z*8-12:Ac had sigmoidal shape in the log10 dose scale and was modeled with a non-linear regression (spike frequency = 57.42/(1 + exp((1.55 − log10(pg))/0.73), r^2 = 0.99), where "pg" is the loading quantity of the stimulus in pg. *N* = 33 for *Z*8-12:Ac, *Z*8-12:OH and for 1 and 10 ng of *E*8-12:Ac. For the other concentrations of *E*8-12:Ac, *N* = 4–16. Average response to hexane = 6.4 ± 1.2 spikes/s (mean ± SEM). (B) *E*8-12:Ac ORNs responded strongly to *E*8-12:Ac, less intensely to *Z*8-12:Ac, and almost no response to *Z*8-12:OH was observed, which was tested only at the two highest concentrations. *N* = 10–14 for all compounds. The response to *E*8-12:Ac was modeled (spike frequency = 50.57/(1 + exp((1.69 − log10(pg))/0.59)), r^2 = 0.99) and this equation was used to subtract the contribution of the 0.38% *E*8-12:Ac in *Z*8-12:Ac from the response of *E*8-12:Ac ORNs to *Z*8-12:Ac. The equations describing the response to *Z*8-12:Ac before and after correction are, respectively, spike frequency = 46.462/(1 + exp((2.67 − log10(pg))/0.61)), r^2 = 0.99, and spike frequency = 21.47/(1 + exp((1.98 − log10(pg1))/0.46)), r^2 = 0.90, where "pg1" refers to the 0.38% *E*8-12:Ac in the amount of *Z*8-12:Ac shown in the *x*-axis. Average response to hexane = 0.14 ± 1.13 spikes/s (mean ± SEM). (C) Representative recordings of one *Z*8-12:Ac ORN (top) and one *E*8-12:Ac ORN (bottom) stimulated with 1 ng of *Z*8-12:Ac or *E*8-12:Ac (200 ms puffs: horizontal bar over the trace).

variable in amplitude and spontaneous activity than the pheromone cells. 60% of the unresponsive cells had large spike amplitudes, as in pheromone cells (>1 mV), whereas the remaining 40% had small spike amplitudes (<1 mV). The spontaneous activity of 73% of the unresponsive cells was high (>10 AP/s), whereas in the remaining 27% the spontaneous activity was comparable with that of pheromone cells (<10 AP/s) (Table S4).

3.2.3. Dose–response

The response of Z-cells to *Z*8-12:Ac and of E-cells to *E*8-12:Ac was sigmoidal in shape in the log-concentration scale, with a response intensity similar to *n*-hexane control up to the 1 pg stimulus concentration, rising steeply up to 1 ng stimulus concentration and starting to balance off at the 10 ng stimulus concentration (Fig. 2). *n*-hexane produced minute changes in spontaneous activity (Fig. 3). When the effect of the 0.38% contamination of *E*8-12:Ac in *Z*8-12:Ac was corrected, the response of E-cells to *Z*8-12:Ac decreased by about half, but it still was comparatively larger than the response of these cells to *Z*8-12:OH, or than the response of Z-cells to *E*8-12:Ac (Fig. 2B).

Electroantennogram recordings showed significantly higher responses to the 3 pheromone components than to *n*-hexane at the highest stimulus concentration tested (1 μg; planned contrasts between each compound and *n*-hexane following a GLM for each concentration, $P < 0.05$; Fig. S3). At the two lower stimulus concentrations tested, the two acetates stimulated the antennae more than hexane, but the response to the alcohol was similar to hexane.

3.2.4. Cross-adaptation

The dual response of E-cells to *E*8-12:Ac and *Z*8-12:Ac could result from a single neuron responding to the two compounds, or from two neurons (of identical spike amplitude) but each responding to a different isomer. A cross-adaptation test in E-cells, showed no response to the second puff in any of the cross-compound combinations tested, which is in agreement with the hypothesis that E-cells consist of just one cell responding to both isomers (Fig. 3A). The cross-adaptation response was indistinguishable from the response to two consecutive puffs of the same isomer. In contrast, in Z-cells a puff of *Z*8-12:Ac following a puff of *E*8-12:Ac produced a response during the second puff, but not during the first puff, as is expected from a single cell responding just to *Z*8-12:Ac (Fig. 3B).

3.2.5. Response duration of Z- and E-cells to Z8-12:Ac

Although both Z- and E-cells responded to *Z*8-12:Ac, the temporal pattern of response was different between them (Fig. 4). After stimulation, spontaneous activity rose sharply to peak frequency,

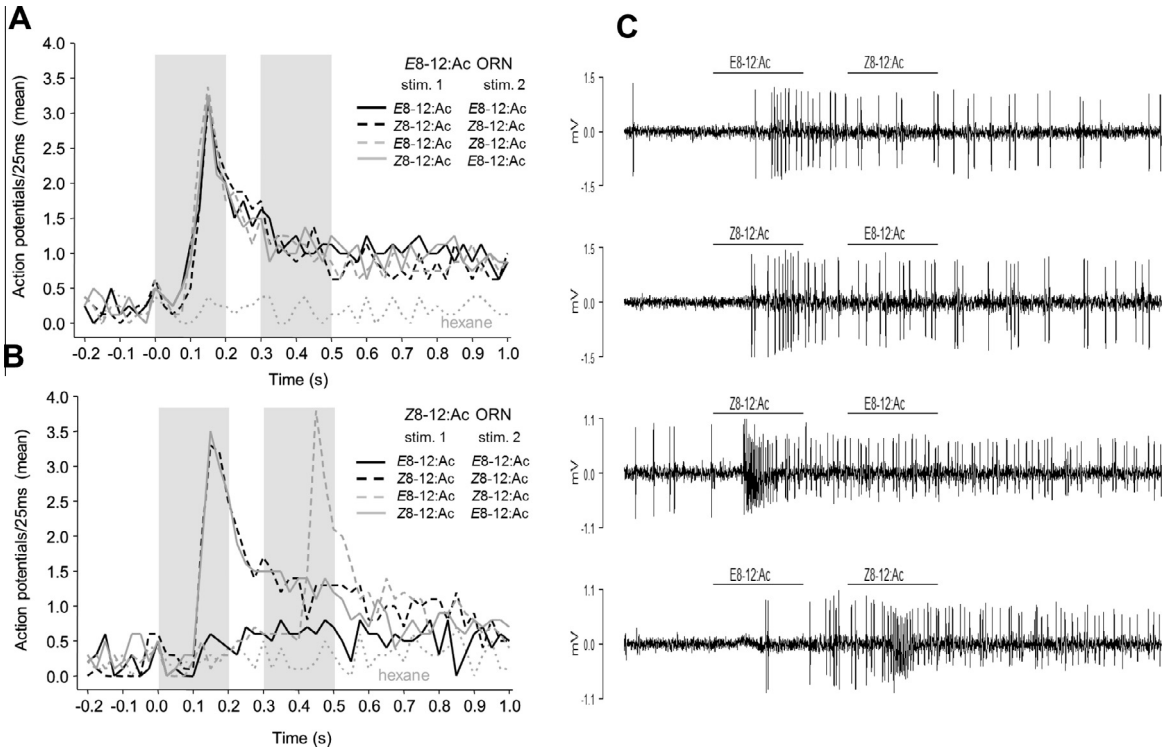

Fig. 3. Cross-adaptation test in *E*8-12:Ac ORNs (A, $N = 8$) and *Z*8-12:Ac ORNs (B, $N = 10$) in *G. molesta* males. ORNs were stimulated with two closely spaced puffs of *Z*8-12:Ac or *E*8-12:Ac (grey bars). The relative frequency of spikes in 25 ms bins is plotted against time. Notice the lack of response to the second stimulation of the opposite stimulus in cross-stimulus tests with *E*8-12:Ac ORNs (A), indicating that the same cell responds to both compounds. By contrast, in the *Z*8-12:Ac ORNs (B) stimulation with *Z*8-12:Ac adapts the cell to *Z*8-12:Ac but stimulation with *E*8-12:Ac does not, as is expected in highly specific ORNs. The loading quantities of *Z*8-12:Ac and *E*8-12:Ac were 100 pg and 1 ng, respectively, in Z-ORNs and the reverse in E-ORNs. Only means are shown for the sake of clarity. (C) Representative traces of the cross-stimulations (two top traces: one *E*8-12:Ac ORN; two bottom traces: one *Z*8-12:Ac ORN). Each horizontal bar represents a 200 ms puff of either *Z*8-12:Ac or *E*8-12:Ac.

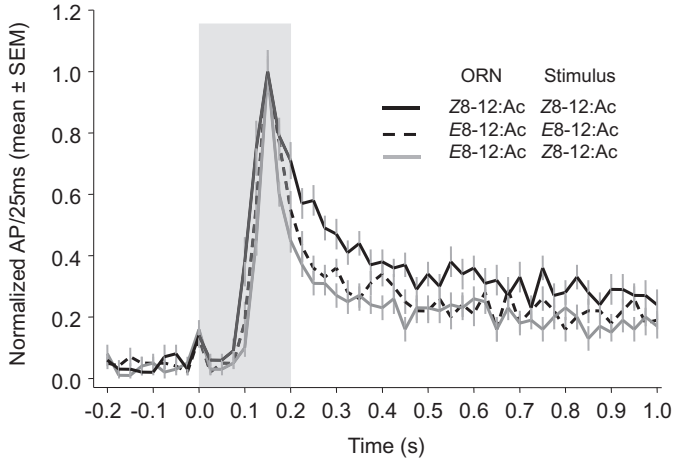

Fig. 4. Response dynamics of *Z*8-12:Ac and *E*8-12:Ac ORNs to stimulation with *Z*8-12:Ac (100 pg in *Z*8-12:Ac ORNs and 1 ng in *E*8-12:Ac ORNs), and of E-ORNs to stimulation with 100 pg of *E*8-12:Ac ($N = 27$–35). Stimulation with *Z*8-12:Ac resulted in a significantly longer-lasting response in *Z*8-12:Ac ORNs than in *E*8-12:Ac ORNs (*t*-test, $P < 0.05$). *E*8-12:Ac ORNs displayed similar dynamics to the two pheromone compounds.

and then decreased to spontaneous activity level in both cell types. The time after puff onset and half-rise was similar in Z- and E-cells (mean ± SEM, 0.148 ± 0.005 s and 0.147 ± 0.002 s, respectively; *t*-test, $P = 0.410$), but Z-cells remained active for a longer time as indicated by a longer time to half-fall in these cells (mean ± SEM, 0.299 ± 0.004 s) than in the E-cells (mean ± SEM, 0.224 ± 0.003 s; *t*-test, $P < 0.001$). *E*8-12:Ac ORNs displayed similar dynamics to the two pheromone compounds.

4. Discussion

4.1. Sensilla morphology

The six morphological sensilla types of *G. molesta* are similar to those reported in other tortricids (Wall, 1978; Razowski and Wojtusiak, 2004; Ansebo et al., 2005), and Lepidoptera in general (Hansson, 1998). The total number of sensilla trichodea that we recorded, however, is much lower than what George and Nagy (1984) report (4382 and 9095, respectively, for the sum of the two antennae). Nagy and George (1981) show that total counts of sensilla trichodea in *G. molesta* vary up to 30% among individuals reared under different conditions, so the different number of sensilla between studies could be related to this factor. The percentage of sensilla trichodea relative to other sensilla types that we observed is similar to the 81% reported by George and Nagy (1984).

Sensilla auricillica and coeloconica were the next most abundant sensilla types on the antennae of *G. molesta* males. They are probably involved in the detection of plant odours and other organic compounds (Ebbinghaus et al., 1997; Ansebo et al., 2005; Wall, 1978). George and Nagy (1984) distinguish two types of sensilla basiconica on the antennae of *G. molesta* males, however we could identify only one type. They mapped the longer type I basiconica to the distal half of the scale-free area, and the shorter type II to the proximal half of the scaled area. In our observations sensilla basiconica were all located in the distal half of the flagellomere, and corresponded in length and number with sensilla basiconica type I of George and Nagy (1984), so they are probably the same type of sensillum. We, however, could not find sensilla basiconica type II of George and Nagy (1984), perhaps because it is shorter than sensilla basiconica type I. The arrangement of four

sensilla chaetica in the equator of the flagellomere and one sensillum styloconicum at the tip is common in other tortricids (Wall, 1978; Ebbinghaus et al., 1997; Maher and Thiery, 2004; Ansebo et al., 2005).

4.2. Distribution of pheromone ORN types

Unlike what we expected, the ORNs tuned to the major pheromone component *Z*8-12:Ac are housed in different sensilla trichodea than the ORNs tuned to the stereoisomer, *E*8-12:Ac. Similar sensilla partition of ORNs is found in some noctuid moths, but in the other tortricids investigated, and in many other moth species, major component ORNs share sensilla with minor component ORNs (reviewed in De Bruyne and Baker, 2008; Baker et al., 2012). It has been proposed that the adaptive function of co-localized ORNs is related to the physiological constraint imposed by real time detection of precise odourant blend ratios (Baker et al., 1998, 2012; Binyameen et al., 2014). The question remains as to why in some species like *G. molesta*, pheromone ORNs are not co-localized, whereas in others like *Ostrinia nubilalis* (Hübner) they share sensillum with other pheromone ORNs (Domingue et al., 2007).

As a general rule, when ORNs are co-localized the major component ORN has a larger dendrite size, whereas sensilla with a single ORN responding to the major component are more abundant than sensilla housing neurons tuned to minor components (Baker et al., 2012). The second case applies to *G. molesta* because major and minor component ORNs occur in different sensilla, and the major component ORNs are more abundant than minor component ORNs, whereas the spike amplitude of both ORN types is relatively similar, which is an indication of similar dendrite size between them (Hansson et al., 1994).

4.3. Pheromone-unresponsive ORNs

A relatively large percentage (29%) of the sensilla trichodea in *G. molesta* males house ORNs that do not respond to the pheromone components. In male *Manduca sexta* (L.) 59% of the sensilla trichodea host ORNs responsive to sex pheromone components, whereas the rest either respond to plant odours (20%) or do not respond to any test compound (21%) (Kalinová et al., 2001). In male *Agrotis segetum* (Schiff.) (Hansson et al., 1989) and *Heliothis subflexa* (Guenée) (Baker et al., 2004) relatively smaller percentages of unresponsive ORNs were reported in sensilla trichodea (11% and 2%, respectively). Male *G. molesta* respond behaviorally to plant volatiles (Varela et al., 2011a; Il'ichev et al., 2009; Lu et al., 2013), so some of their unresponsive ORNs could be tuned to plant volatiles. In addition, ORNs unresponsive to pheromone compounds could be used to detect pheromone compounds from other species that inhibit male *G. molesta* response to the female pheromone, such as *Z*6-12:Ac and *Z*10-14:OH (Guerin et al., 1986; Tòth et al., 1991), as happens in other species (reviewed in De Bruyne and Baker, 2008).

4.4. Ligand specificity of pheromone ORNs

Male *G. molesta* behaviorally discriminate small variations in the ratio of the two acetate isomers (Baker and Cardé, 1979; Baker et al., 1981; Knight et al., 2014a), so they should have a detection system that reports the relative abundance of the two isomers in the blend. In the majority of moths this is achieved by having specific receptor neurons to each of the main pheromone components (De Bruyne and Baker, 2008). However in *G. molesta* the ORNs tuned to the minor component (*E*8-12:Ac) also respond to the major component (*Z*8-12:Ac). The 0.38% *E*8-12:Ac contamination in the *Z*8-12:Ac solution contributed only slightly to the unspecific response of the E-cells, as we demonstrated after subtracting its effect. The cross-adaptation test indicated that sensilla housing the E-cell did not house a second ORN responding to *Z*8-12:Ac, and confirms that a single and not very specific ORN responds strongly to *E*8-12:Ac and less to *Z*8-12:Ac.

Low specificity ORNs for key pheromone components have been scarcely reported in the literature (Takanashi et al., 2006; Domingue et al., 2008). In *Ostrnia furnacalis* (Guenée) a large proportion of the ORNs respond equally well to the two main components (*E*12-14:OAc and *Z*12-14:OAc) (Takanashi et al., 2006). However, unlike *G. molesta, O. furnacalis* also has ORNs specifically tuned to each pheromone component. So, how to explain the apparent absence of *E*8-12:Ac-specific ORNs in *G. molesta*? One possible explanation is that *E*8-12:Ac-specific ORNs are rare and were missed in our sample of 176 sensilla. Very low frequencies (<2%) of ORNs tuned to pheromone components have been reported in other species (Hansson et al., 1990; Quero et al., 2004).

Another possibility is that fully specific ORNs are not essential for pheromone blend discrimination in *G. molesta*. To explain this point we must assume that each ORN type (Z and E) innervates a different glomerulus, as happens in most moth species (Lei and Vickers, 2008), and that both glomeruli will be excited by *Z*8-12:Ac, but more intensely the *Z*8-12:Ac than the *E*8-12:Ac glomerulus, due to the larger number of *Z*8-12:Ac ORN axons innervating it. Because *E*8-12:Ac will excite only the *E*8-12:Ac glomerulus, departures in the relative response of the two glomeruli to a blend of both isomers, with respect to excitation with *Z*8-12:Ac alone, will inform the insect of the presence of *E*8-12:Ac in the blend. Differential glomerular excitation could, thus, report stimulus composition even when one of the two ORNs is not fully specific. To confirm this point we should determine the presence of specific glomeruli for the Z- and E-ORNs. Male *G. molesta* have one large glomerulus at the entrance of the AL that is lacking in females (Valera et al., 2009), so it is very likely that this glomerulus is innervated by *Z*8-12:Ac-specific ORNs. Antennal retrograde staining coupled with electrophysiological recordings (Hansson et al., 1992) could confirm if each ORN type innervates a different glomerulus, and calcium imaging (Piñero et al., 2007) could measure the relative response ratio of the glomeruli to different pheromone blends.

The different temporal response dynamics of Z- and E-cells in response to *Z*8-12:Ac that we observed (longer lasting response in Z-cells), could provide odor identity information to the brain, but how this could help the brain to discriminate between Z-and E-excitation in E-cells is not clear.

Although it is generally accepted that pheromone receptor neurons are highly specific (reviewed by De Bruyne and Baker, 2008), relatively few studies have determined the specificity of the most behaviorally relevant pheromone component ORNs for a given species. Additional studies of ORN specificity are needed to determine if species bearing low-specificity pheromone ORNs, like *O. furnacalis* and *G. molesta*, are more common than generally assumed.

4.5. Detection of *Z*8-12:OH

One unexpected result from our study was the apparent absence of Z8-12:OH-specific ORNs because this compound is emitted by females and affects male attraction (Cardé et al., 1975a, 1979; Baker and Cardé, 1979; Baker et al., 1980; Linn and Roelofs, 1983), and in our EAG tests it stimulated the antenna. *Z*8-12:OH is 10% of the pheromone blend, so it probably has few dedicated ORNs that were missed in our sampling of 176 sensilla, or these neurons were not very specific or housed in sensilla other than trichodea (e.g., sensilla auricillica in *Cydia pomonella* (L.), Ebbinghaus et al., 1997; Ansebo et al., 2005), which were not sampled in our study. Whereas narrow variations in the ratio of the two acetates have a strong effect in male behavioral response

(Baker et al., 1981; Knight et al., 2014a), males accept wide variations in the quantity of *Z*8-12:OH in the blend (Baker and Cardé, 1979; Linn and Roelofs, 1983; Linn et al., 1986), and in some locations *Z*8-12:OH is produced in trace amounts by females (Lacey and Sanders, 1992), or does not seem to play a role in attraction (Han et al., 2001; Jung et al., 2013). Furthermore, other alcohols affect the response of male *G. molesta* to the two acetates (Baker and Cardé, 1979; Cardé et al., 1975a,b, 1979), including the sex pheromone of *C. pomonella* (Knight et al., 2014b), and *Z*8-12:OH inhibits males of closely related species (*Grapholita funebrana* (Treitschke) and *Grapholita prunivora* (Walsh)), that use a similar ratio of the Z/E acetates as *G. molesta* (Baker and Cardé, 1979; Guerin et al., 1986), so a reinvestigation of the role of alcohols in the olfactory communication of *G. molesta* is warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2014.10.011.

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Response profile of pheromone receptor neurons in male *Grapholita molesta* (Lepidoptera : Tortricidae)

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Supplementary Material

Sensillum type	Length	Basal width	Tip width
Trichodea	38.86 ± 0.28	1.63 ± 0.02	0.42 ± 0.00
Auricillica	13.73 ± 0.46	1.97 ± 0.02	1.86 ± 0.03
Coeloconica[1]	6.09 ± 0.07	1.16 ± 0.05	0.27 ± 0.01
Basiconica	12.86 ± 0.37	1.70 ± 0.02	0.77 ± 0.05
Chaetica	31.68 ± 0.02	1.59 ± 0.02	0.49 ± 0.00
Styloconica	19.25 ± 0.23	3.68 ± 0.07	2.51 ± 0.04

Table S1. Morphometry (μm ± SEM) of sensilla types on male *G. molesta* antennal flagellum (N=20). [1] In coeloconica only measured the central part.

Supplementary material

Sensillum type	Scaled area (n=9)		Scale-free area (n=4)		Total	
	number	%	number	%	number	%
Trichodea	1,015.56 ± 78.71	65.89 ± 1.20	1,276 ± 42.84	78.63 ± 0.09	2,291.5	72.11
Auricillica	294.67 ± 22.59	19.13 ± 0.61	53.75 ± 11.25	3.28 ± 0.62	348.42	10.96
Coeloconica	127.77 ± 16.98	8.02 ± 0.63	95 ± 4.56	5.86 ± 0.31	222.78	7.01
Basiconica	36.67 ± 6.48	2.38 ± 0.44	76.25 ± 4.73	4.71 ± 0.33	112.92	3.55
Chaetica	80.55 ± 2.94	5.36 ± 0.25	81.75± 4.66	5.02 ± 0.12	162.31	5.18
Styloconica	0	0	40 ± 2.04	2.46 ± 0.09	40	1.26
Total	1,534.38 ± 114.09		1,622.75 ± 55.66		3,168.08	

Table S2. Number (mean ± SEM) of morphological sensillum types on scaled and scale-free areas of the antennal flagellum of *G. molesta* males. Sum of scaled and scale-free areas is not averaged because counts from scaled and scale-free areas come from different sets of antennae.

Sensillum type	ORN/ sensillum	Scaled area	Scale-free area	Total (%)
Z-type	1	48	38	86
	2	0	19	19
	3	0	7	7
	Total (%)	48/63 (76.1%)	64/113 (56.6%)	112/176 (63.6%)
E-type	1	4	3	7
	2	3	3	6
	3	0	0	0
	Total (%)	7/63 (11.1%)	6/113 (04.4%)	13/176 (07.4%)
Unresponsive	1	5	12	17
	2	3	9	12
	3	0	22	22
	Total (%)	8/63 (12.6%)	43/113 (38.0%)	51/176 (28.9%)

Table S3. Number of sensilla trichodea types in the antennal flagellum of *G. molesta* males according to the number of ORNs present per sensillum (1, 2 or 3), the response of the ORNs (Z, E or unresponsive), and their location (scaled or scale-free areas) (N = 20 and 39 individuals, respectively). Z and E-ORNs were housed in separate sensilla. Notice the high proportion of pheromone unresponsive sensilla (28.9%), and the larger number of Z-cells housed in 2-ORN sensilla in the scale-free area than in the scaled area. The ratio of Z:E cells (100:11.6) corresponds approximately to the ratio of these pheromone components in the pheromone blend (100:6).

Cell type	ORN/ sensillum	Amplitude (mV)	Spontaneous activity (AP/sec)	Respond to pheromone
Z-type	1	1.81 ± 0.17	7.44 ± 0.84	25/25
	2	1.78 ± 0.22	4.41 ± 0.65	12/12
		0.83 ± 0.19	13.33 ± 3.05	0/12
	3	2.79 ± 0.32	2.57 ± 1.44	3/7
		1.72 ± 0.26	7.57 ± 1.08	4/7
		0.79 ± 0.16	16.28 ± 4.88	0/7
E-type	1	1.57 ± 0.09	7.82 ± 0.84	23/23
	2	1.73 ± 0.26	5.5 ± 1.05	6/6
		0.47 ± 0.06	25.66 ± 14.79	0/6
Unresponsive	1	1.15 ± 0.14	11.0 ± 2.9	0/16
	2	1.54 ± 0.19	5.83 ± 1.96	0/12
		0.79 ± 0.15	22.41 ± 6.87	0/12
	3	2.02 ± 0.24	5.41 ± 1.92	0/12
		1.17 ± 0.19	17.16 ± 4.06	0/12
		0.63 ± 0.14	14.66 ± 3.32	0/12

Table S4. Spike amplitude and spontaneous activity of ORNs in male *G. molesta* antennae according to their response type (Z, E and unresponsive) and to the number of ORNs per sensillum. The ORNs in Table S4 include some ORNs from Table S3.

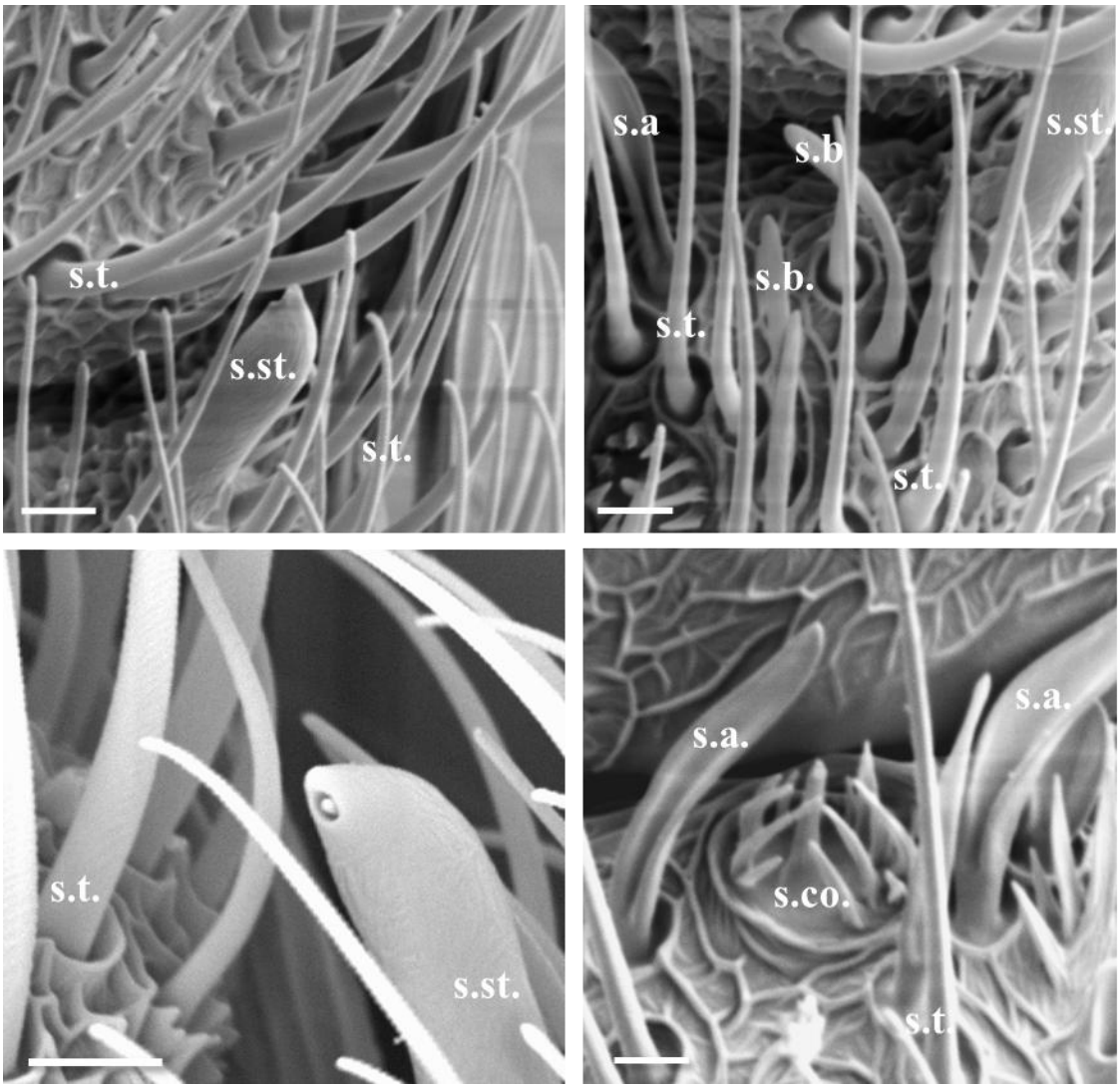

Figure S1. Different types of sensilla on the antennae of male *G. molesta*. Sensilla auricillica (s.a), sensilla basiconica (s.b), sensilla coeloconica (s.co), sensilla styloconica (s.st.), and sensilla trichodea (s.t). Scale bar = 5 µm.

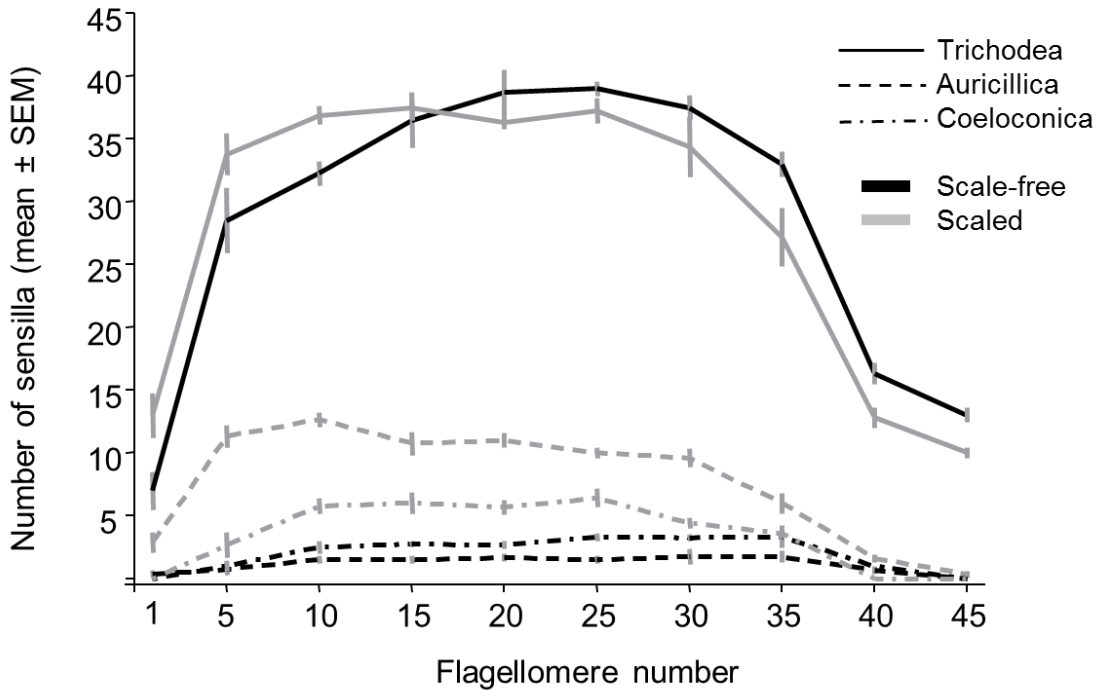

Figure S2. Abundance of three sensilla types (trichodea, auricillica and coeloconica) along the antennal flagellum of *G. molesta* males.

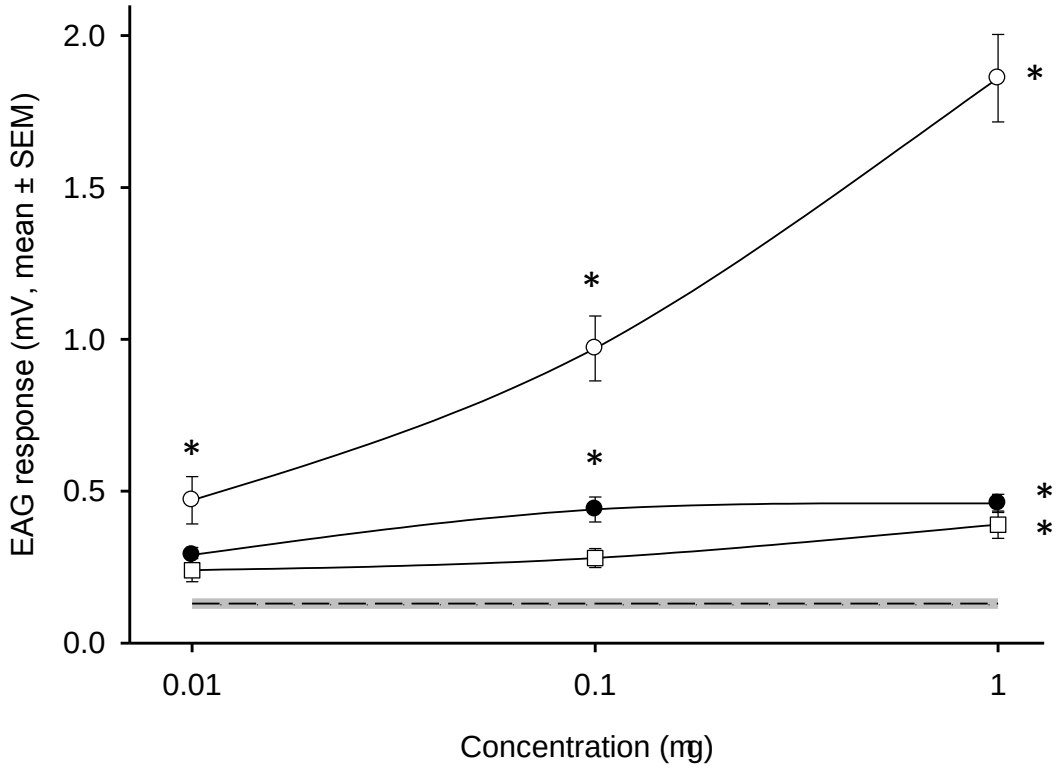

Figure S3. Electroantennographic responses of male *G. molesta* antennae to the three pheromone components. Asterisk indicates difference with hexane within a concentration (GLM followed by planned contrasts, $P < 0.05$)