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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.