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(1*S*,3*S*,7*R*)-3-methyl-α-himachalene from the male sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae) induces neurophysiological responses and attracts both males and females

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Abstract

Lutzomyia longipalpis adult males form leks on or near hosts and release (1*S*,3*S*,7*R*)-3-methyl-α-himachalene from their tergal glands to lure females to the same site for mating and feeding. Here we have examined whether the male-produced attractant could also serve as a male aggregation stimulus. High resolution chiral capillary gas chromatography analysis of male tergal gland extracts, synthetic (1*S*,3*S*,7*R*)-3-methyl-α-himachalene, and a synthetic mixture of all isomers of 3-methyl-α-himachalene, was coupled to electrophysiological recordings from ascoid sensillum receptor cells in antennae of male and female sandflies. Receptor cells of both sexes responded only to the main component of the male tergal gland extract that eluted at the same retention time as (1*S*,3*S*,7*R*)-3-methyl-α-himachalene. Furthermore, of the eight 3-methyl-α-himachalene isomers in the synthetic mixture only the fraction containing (1*S*,3*S*,7*R*)-3-methyl-α-himachalene, co-eluting with an isomer of (1*S**,3*S**,7*S**)-3-methyl-α-himachalene, elicited an electrophysiological response from male and female ascoid sensillum receptor cells. Both males and females flew upwind in a wind tunnel towards a filter paper disk treated with either 4–6 male equivalents of the tergal gland extract, pure (1*S*,3*S*,7*R*)-3-methyl-α-himachalene or the synthetic mixture of eight isomers. This indicates that (1*S*,3*S*,7*R*)-3-methyl-α-himachalene derived from *L. longipalpis* males may have a dual function in causing male aggregation as well as serving as a sex pheromone for females.

Keywords: Sandfly; 3-methyl-α-himachalene; Pheromone; Electrophysiology; Lek behaviour

1. Introduction

The sandfly *Lutzomyia longipalpis* (Lutz and Neiva, 1912; Diptera: Psychodidae) is the main vector of visceral leishmaniasis in Latin America (Grimaldi et al., 1989), which is caused by *Leishmania chagasi*/*L. infantum* (Protozoa: Kinetoplastida). The global annual incidence of this disease is estimated to be 0.5 million cases, the great majority in Bangladesh, Brazil, India and Sudan (Desjeux, 1996). *L. longipalpis* represents a complex of sibling species in which mate recognition appears to be mediated by the specificity of the sex pheromone. *L. longipalpis* males release a sex pheromone from glands clustered in pale patches on the fourth, or alternatively, the third and fourth abdominal tergites (Lane and Ward, 1984). Chemical analysis of the pheromone gland extract revealed at least three different types of sex pheromone produced by Brazilian *L. longipalpis* sibling species, depending on their geographical location (Hamilton et al., 1996a, b; 2002): The homosesquiterpene (1*S*,3*S*,7*R*)-3-methyl-α-himachalene is specific to *L. longipalpis* from Jacobina, Bahia State, N.E.

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Brazil, while another homosesquiterpene, (*S*)-9-methylgermacrene-B is specific to *L. longipalpis* from Lapinha Cave, Minas Gerais, S.E., Brazil and a putative diterpene (C20) specific to *L. longipalpis* from Sobral State, Ceará State, N.E. Brazil. Laboratory experiments indicate that Brazilian populations of *L. longipalpis* respond to the male pheromone in a sibling species-specific manner (Ward and Morton, 1991). The sex pheromone gland of *L. longipalpis* from Jacobina produces several compounds, but only the main component, (1*S*,3*S*,7*R*)-3-methyl-α-himachalene, that constitutes 90% of the gland extract has been demonstrated to be responsible for attraction of females (Hamilton et al., 1994). Mori et al. (2000) assigned the (1*S**,3*S**,7*R**)-3-methyl-α-himachalene relative stereochemical configuration to the pheromone, and the absolute stereochemistry was assigned after an enantioselective synthesis was developed (Tashiro et al., 2000; Mori et al., 2000).

Volatile semiochemicals are perceived via olfactory receptors in ascoid sensilla on the antennae of *L. longipalpis* (Dougherty et al., 1995; 1999). Ascoids are paired structures found on the 3rd–15th antennal segments, in both sexes (Boufana, 1990). Dougherty et al. (1995) made the first electrophysiological recordings from these ascoid sensilla on female antennae. They reported neurones sensitive to compounds from host faecal material, to a component of the sex pheromone, to an oviposition stimulant and to compounds from the odour-producing glands of the fox *Vulpes vulpes* (Dougherty and Hamilton, 1997; Dougherty et al., 1999).

Host odour has been shown to increase the attraction of females to the male-produced sex pheromone (Morton and Ward, 1989; Nigam and Ward, 1991). Males are also attracted by host odours (Hamilton and Ramsoondar, 1994) even though they are not haematophagous. Sandflies of the *L. longipalpis* species complex form nocturnal leks on or near vertebrate hosts (Quinnel and Dye, 1994; Kelly and Dye, 1997, Jones and Hamilton, 1998) where they attract females to a blood meal thus increasing their chances of mating. In the laboratory, leks can be even established in small net cages with or without a host (Jarvis and Rutledge, 1992).

(1*S*,3*S*,7*R*)-3-methyl-α-himachalene has so far been considered only as a sex pheromone (Hamilton et al., 1994; Ward et al., 1989). Here we hypothesize that this compound could also serve to attract other *L. longipalpis* males to the lek. Electrophysiological and behavioural responses were recorded from males and females to the sex pheromone gland extract, to synthetic (1*S*,3*S*,7*R*)-3-methyl-α-himachalene and to a synthetic mixture containing all eight isomers of 3-methyl-α-himachalene.

2. Materials and methods

2.1. Insects

Sandflies used in this study were from Jacobina, Bahia State, Northeastern Brazil, supplied by Professor R. Ward, Keele University, UK. They were reared according to the method of Modi and Tesh (1983) at a temperature of 28 °C, 90 ± 5% RH, 14:10 L:D cycle, with 3 h light ramps at dawn and dusk. Sandflies used for electrophysiological recordings were 2–3 day-old and were not blood-fed. For the wind tunnel experiments, males and females were isolated up to 12 h after emergence and were maintained in a cage (10 cm × 10 cm × 10 cm) or in a plastic pot (500 cm^3) covered with gauze disposed in the same environmental chamber as the wind tunnel at 28 C, 80% RH, 14 h day (115 LUX) and 22 °C, 85% RH, 10 h night, with 1 h light ramps and 2 h T and RH ramps at dawn and dusk. Male and female 4–5 day-old sandflies were tested individually in the wind tunnel to avoid any intraspecific communication during tests. The insects were deprived of sugar solution at least 1 h before testing in the wind tunnel and no more than 10 insects were tested per evening.

2.2. Pheromone extract

The 3rd and 4th abdominal tergites housing the pheromone glands in males (Lane and Ward, 1984) from 4–5 day-old flies were dissected into a tapered glass vial (1.1 ml, Chromacol, UK) held on dry ice with 5 μl hexane (p.a., Merck, Zürich, Switzerland) added per male. Males of this age are known to have their glands replete with pheromone (Boufana, 1990). The gland extract from 5 to 20 males was then stored at −21 °C as recommended by Ward et al. (1989).

2.3. Synthetics

Synthetics used for electrophysiological and behavioural assays were (1*S*,3*S*,7*R*)-3-methyl-α-himachalene (Mori et al., 2000) and a synthetic mixture containing eight isomers of 3-methyl-α-himachalene (Fig. 1) in the following proportions: 4% (1*S*,3*S*,7*R*)-, 4% (1*R*,3*R*,7*S*)-, 11% (1*S*,3*S*,7*S*)-, 11% (1*R*,3*R*,7*R*)-, 3% (1*S*,3*R*,7*S*)-, 3% (1*R*,3*S*,7*R*)-, 32% (1*S*,3*R*,7*R*)-, 32% (1*R*,3*S*,7*S*)-.

2.3. Electrophysiology

The method of recording electrophysiological responses from *L. longipalpis* male and female ascoid sensilla receptor cells and the stimulus delivery system were as described by Dougherty et al. (1995; 1999). Responses were recorded from different ascoids along the antenna and the tungsten electrode was always inserted near the ascoid base. The recording electrode was etched to a diameter of ⩽1μm. The antenna was held in a humidified air-stream (90–100% RH) delivered at 1 ms^{-1} via a glass water-jacketed tube (6 mm i.d.) with its outlet approximately 1 cm away from the preparation. The electrophysiological signal was fed into an AC/DC amplifier (UN-03, Syntech, The Netherlands) via a high-impedance preamplifier (Syntech), recorded on the hard disk of a PC via a 16-bit analog/digital IDAC box (Syntech) and monitored simultaneously with

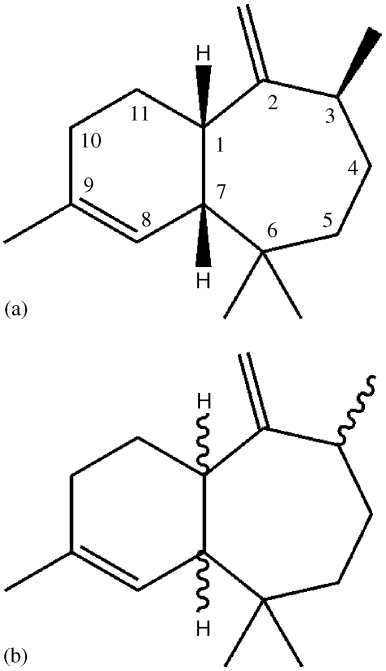

Fig. 1. Chemical structure of (a) the synthetic (1*S*,3*S*,7*R*)-3-methyl-α-himachalene and (b) the synthetic mixture containing eight isomers of 3-methyl-α-himachalene.

an oscilloscope (Tektronix 5103, USA). Recordings were analysed with Autospike software (version 3.0, Syntech).

Samples of 0.1–10 male equivalents of the pheromone extract were deposited on a filter paper strip (0.8 cm × 3 cm) and placed in the 5 ml stimulus syringe after evaporation of the solvent. The 1 s stimulus puff was introduced from this syringe through a septum-covered hole (24 cm from the preparation) in the water-jacketed tube. α-Pinene (1 μg on filter paper in the stimulus syringe) was used to control the state of the preparation as the ascoid sensilla have been previously shown to contain a receptor cell responding to α-pinene (Dougherty et al., 1995).

2.4. Gas chromatography-linked ascoid sensillum recordings (GC-SSR)

Components of the male sex gland extracts and six of the eight isomers of synthetic 3-methyl-α-himachalene could be separated by high resolution gas chromatography (one of the (1*S**,3*S**,7*S**)- isomers co-eluted with the (1*S*,3*S*,7*R*)-isomer), using a chiral capillary column (30% 6-*O*-*t*-butyldimethylsilyl-2,3-*O*-dimethyl-β-cyclodextrin in polysiloxane-086, 25 m, 0.32 mm i.d., 0.25 μm film thickness; Institute of Chemistry, University of Neuchâtel) installed in a Carlo Erba Instruments 5300 gas chromatograph with an on-column injector and a flame ionization detector (FID) set at 230 °C. H_2 was used as carrier gas. Two different temperature programmes were used for separations: (1) injection at 60 °C for 5 min, 5 °C min^{-1} to 200 °C, and (2) injection at 100 °C for 25 min for isothermal separation of the enantiomers. 0.2 male equivalents of the pheromone gland extract containing approximately 60 ng of the biologically active component and 100 ng of either the synthetic (1*S*,3*S*,7*R*)-3-methyl-α-himachalene or the synthetic mixture of eight isomers of 3-methyl-α-himachalene were injected in the GC. The column effluent was split equally (Gerstel splitter, Mühlheim an der Ruhr, Germany) between the FID and the electrophysiological preparation. A heated transfer line (220 °C) assured volatiles exiting the GC be swept by the humidified air-stream over the electrophysiological preparation. Action potentials recorded from ascoid sensilla, employed as a biological detector, were sorted by a level discriminator incorporated in the UN-03 amplifier, and the sum of the frequencies of all firing cells was continuously converted to a voltage (1 s time constant). The AC spike signal, the DC signal (representing the sum of the spikes) and the FID response were recorded simultaneously on a computer (Autospike, Syntech).

2.5. Wind tunnel

The wind tunnel (170 cm long 60 cm × 60 cm) made of non-reflecting glass had a laminar airflow at 30 cm s^{-1}. Overhead illumination with fluorescent tubes (36 W, >1 kHz, Philips, Zürich, Switzerland) ran the length of the tunnel (Syed and Guerin, 2004). The sandflies were flown during the early scotophase, as lek-like aggregations start to form at dusk in nature. In the wind tunnel the light intensity was 0.02 lux on the floor. As the sandflies show a hopping flight behaviour habit, a 1.5 m long × 20 cm diam. nylon net (1 mm) flight cylinder was stretched 21 cm above the tunnel floor encompassing the odour plume.

The insects were placed in a transparent release cage (4 cm diam. × 12.5 cm long) with the down- and upwind end covered with nylon gauze (0.063 mm^2 mesh). This cage was set up at 29 cm above the floor of the tunnel. The upwind end of the cage had a vertical sliding door that was attached to a string on a pulley on the roof of the wind tunnel to permit opening from outside.

The pheromone extract was mixed with paraffin oil (1:1) to diminish evaporation during experiments. Four to six male equivalents were placed on a filter paper disk (45 mm diam.) at least 30 min before use. Synthetic (1*S*,3*S*,7*R*)-3-methyl-α-himachalene or the synthetic mixture were mixed with paraffin oil (1:1) and tested at doses of 1–6 μg. The control disk was treated with a similar volume of hexane and paraffin oil (1:1). The filter paper disk was held vertical to the airflow on a glass rod 33 cm above the floor of the wind tunnel at the upwind end of the flight cylinder.

For tests with the pheromone gland extract, the sandflies were allowed a 2 min adaptation period in the release cage placed in the wind tunnel. The cage was then slowly opened to avoid mechanical disturbance and flies were exposed for 2 min to a control disk. After this, the control filter paper disk was lowered from beneath the floor of the wind tunnel and the sandflies were presented for 2 min with the gland extract on a second filter paper lifted on a second glass rod. Insects that flew more than 1 m upwind during the control

period were not considered in the test. This sequence was used in order to reduce the bias caused by mechanical disturbances when opening the cage. In all cases, switching from control to stimulus filter paper disks was made without opening the wind tunnel. To fully control for any influence of the test sequence, a second control was made, where the sandflies were allowed 2 min of adaptation to the wind tunnel conditions, 2 min exposure to a blank filter paper disk (to control for any visual stimulation caused by lifting it into place) followed by a second exposure for 2 min to the control filter paper disk impregnated with hexane and paraffin oil. The activity of flies during the period of exposure to the control disc was compared to the activity of flies during exposure to the pheromone gland extract on the filter paper disk presented in the same sequence. Lifting the filter paper disks to the level of insect flight within the tunnel caused no activation of the flies.

For tests with synthetic (1*S*,3*S*,7*R*)-3-methyl-α-himachalene and the synthetic mixture of eight isomers, individual flies were exposed, after 2 min of adaptation, either to the test or the control filter paper disks. The effect of treatments was measured as the percentage of activated sandflies, i.e. flies that walked, hopped or flew less than 50 cm upwind, and the percentage of sandflies attracted to the odour source, i.e. flies that flew more than 50 cm upwind or that passed the stimulus source. We considered 50 cm an appropriate distance to define attraction as it represents the maximal distance reached by the sandflies during the control period. These results were compared using Fisher's exact test.

3. Results

Volatiles from the male pheromone gland extract injected into the air stream flowing over the electrophysiological preparation elicited similar electrophysiological responses from receptor cells within the ascoid sensillum of males and female sandflies (Fig. 2). Different doses of male sex gland equivalents were tested and an electrophysiological response in ascoid receptor cells from 5 females and 6 males was already discernible with 0.1 male equivalents on the filter paper in the stimulus syringe (data not shown).

3.1. GC linked ascoid sensillum analysis of the pheromone gland extract

The chiral gas chromatography analysis of the sex pheromone gland constituents yielded only one major component that elicited responses from neurones within the ascoid sensilla of males and females (Fig. 3). Both males and females showed a sustained response of only one receptor cell in the ascoid sensillum to this constituent that had the same retention time as (1*S*,3*S*,7*R*)-3-methyl-α-himachalene. Despite the variability in the responses recorded from the different ascoid sensillum preparations, all were characterized by a long lasting response sometimes up to 10 min (Fig. 4). The FID response indicates that one male equivalent corresponds approximately to 300 ng of the biologically active component in our extract.

3.2. GC linked ascoid sensillum analysis of the pheromone analogues

Responses of male and female ascoid sensillum receptor cells to synthetic (1*S*,3*S*,7*R*)-3-methyl-α-himachalene eluting from the chiral column were similar to those obtained to the constituent of the pheromone gland extract (Fig. 5). Furthermore, the responding receptor cell also manifested a sustained response sometimes up to 10 min. As with the biologically active constituent of the sex gland extract, it was usually the cell with the biggest amplitude that responded to the synthetic pheromone. In analyses of the synthetic mixture of eight isomers of 3-methyl-α-himachalene a

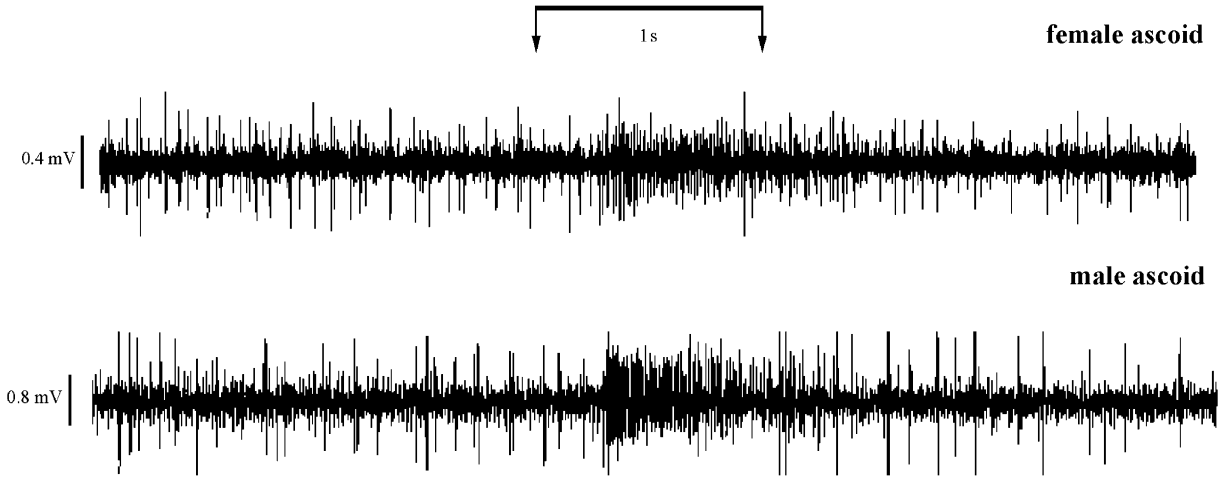

Fig. 2. Action potentials recorded from olfactory receptor cells within ascoid sensilla on the antenna of a female and male *L. longipalpis* to a male sandfly tergal gland extract. The products evaporating from 10 male equivalents in the stimulus cartridge were delivered to the sensillum preparation in 1 s (within arrows). The extract was tested on one ascoid sensillum of 7 females and 10 males, all showing similar action potential bursts.

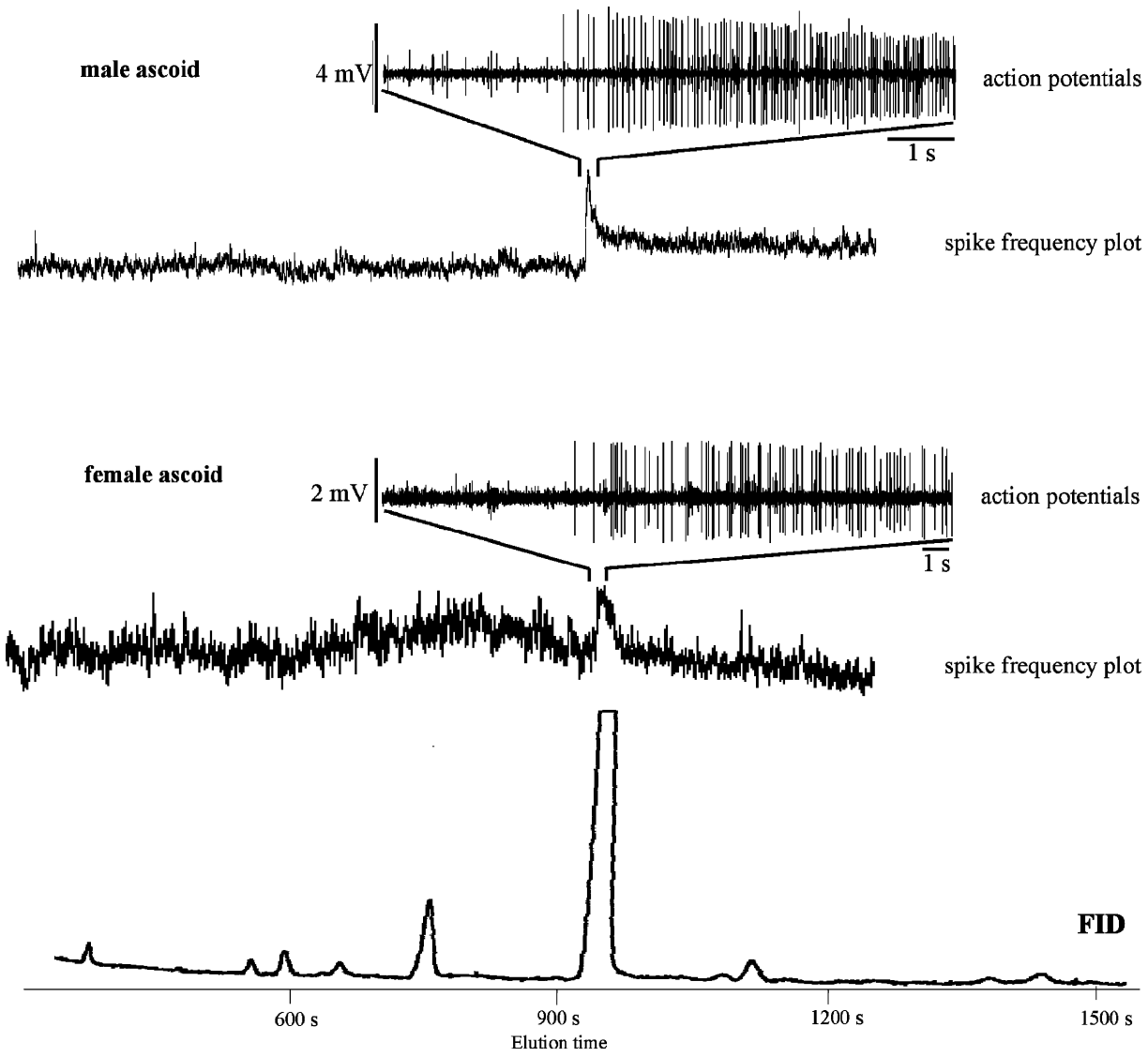

Fig. 3. Analysis of male *L. longipalpis* tergal gland extract on a chiral capillary gas chromatography column-coupled electrophysiological recordings from *L. longipalpis* ascoid sensilla receptor cells of a male and female sandfly. The lower trace is the FID response. The spike frequency plot is the summed frequency of all firing cells after frequency to voltage conversion of the burst in action potentials (expanded upper trace in each case) that accompanied elution of only one biologically active product. The spikes trains depicted represent less than 1% of the response that invariably lasted for at least 5 min. Responses to a single component of the pheromone gland extract eluting at 942 s were recorded from receptors cells in ascoid sensilla of 3 females and 8 males.

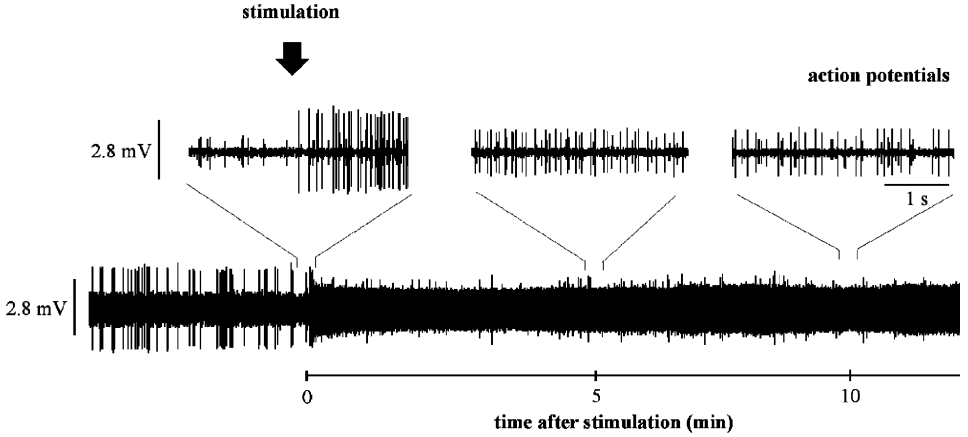

Fig. 4. Illustration of a sustained response from olfactory receptor cells in male *L. longipalpis* ascoid sensilla to a male tergal gland extract on a chiral capillary gas chromatography column-coupled electrophysiological recordings. The arrow indicates the start of (1*S*,3*S*,7*R*)-3-methyl-α-himachalene elution (see Fig. 3). The response lasted several minutes; detailed spikes trains depicted are for 0, 5 and 10 min after stimulation.

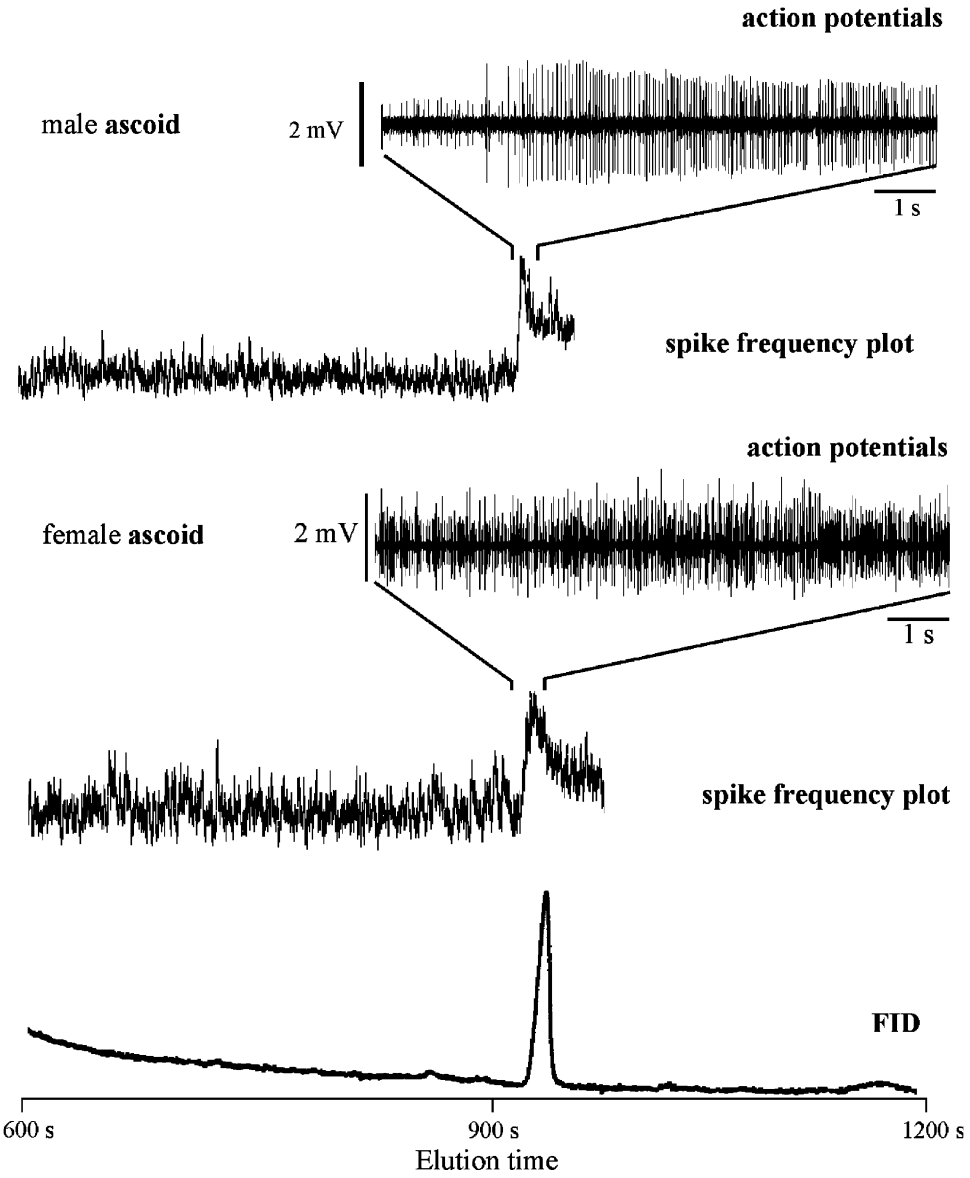

Fig. 5. Responses of male and female *L. longipalpis* ascoid sensillum receptor cells to (1*S*,3*S*,7*R*)-3-methyl-α-himachalene eluting from a chiral capillary chromatography column with a retention time of 930 s. The lower trace is the FID response. The spike frequency plot is the summed frequency of all firing cells after frequency to voltage conversion of the burst in action potentials (expanded upper trace in each case). Similar responses were recorded from receptors cells in ascoid sensilla of 2 females and 2 males.

response was only obtained from the ascoid receptors cells of male and female *L. longipalpis* to the fraction containing (1*S*,3*S*,7*R*)-3-methyl-α-himachalene, co-eluting with one (1*S**,3*S**,7*S**)-3-methyl-α-himachalene isomer (Fig. 6). Co-injection confirmed that this synthetic fraction coeluted with the biologically active sex gland constituent. These data demonstrate the similarity in the sensory responses of the olfactory receptor cells of *L. longipalpis* males and females to 3-methyl-α-himachalene isomers.

3.3. Behavioural responses of male and female sandflies to (1S,3S,7R)-3-methyl-α-himachalene

In the wind tunnel both male and female *L. longipalpis* were activated and attracted to the male tergal gland pheromone extracts exposed on filter paper disks (Table 1a). These experiments revealed that males were better activated ($p < 0.05$, Fisher's exact test) and more attracted ($p < 0.001$, Fisher's exact test) to the sex gland extract than females. The synthetic pheromone (1*S*,3*S*,7*R*)-3-methyl-α-himachalene as well as the synthetic mixture attracted *L. longipalpis* males and females (Table 1b). There was no significant difference in the attraction to synthetic (1*S*,3*S*,7*R*)-3-methyl-α-himachalene and to the synthetic mixture by males and females.

4. Discussion

Our data demonstrate that receptor neurones in the ascoid sensillum on the antennae of *L. longipalpis* males

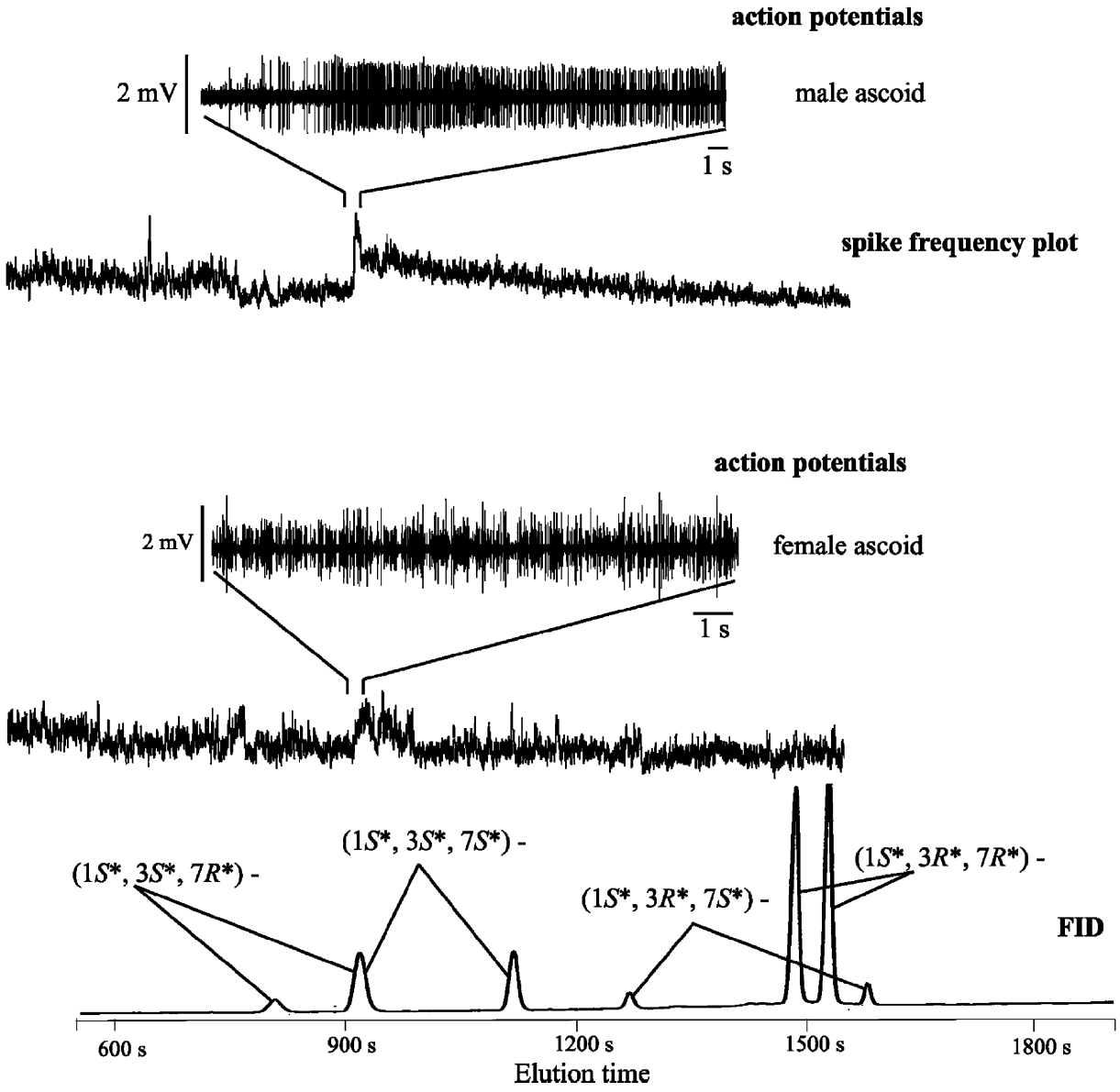

Fig 6. Responses of male and female *L. longipalpis* ascoid sensillum receptor cells to the eight isomers of 3-methyl-α-himachalene as they eluted from a chiral capillary chromatography column. The lower trace is the FID response. The spike frequency plot is the summed frequency of all firing cells after frequency to voltage conversion of the burst in action potentials (expanded upper trace in each case). Responses were recorded from receptors cells in ascoid sensilla of 2 females and 4 males to co-eluting (1*S*,3*S*,7*R*)- and one isomer of (1*S**,3*S**,7*S**)-3-methyl-α-himachalene at 918 s.

respond to a male-produced tergal gland constituent. The electrophysiological response from ascoid sensillum receptor cells of this species to the tergal gland extract that apparently affects the activity of only one cell type had already been described for females (Dougherty et al., 1995). Nevertheless, this is difficult to state with certainly as quite some variability was observed in the responses recorded. This variability could be explained by the fact that the point of insertion of the recording electrode in successive recordings varied in distance from the responding neurone.

The sex pheromone gland of *L. longipalpis* from Jacobina in northeastern Brazil produces several compounds, but only the principal component, (1*S*,3*S*,7*R*)-3-methyl-α-himachalene, that constitutes 90% of the gland extract, is responsible for females attraction (Hamilton et al., 1994). Here we show by chiral gas chromatography-linked ascoid sensillum electrophysiology recordings that receptor cells of male and female ascoid sensilla respond only to the major component of the sex gland extract. This gland component had the same retention time as a biologically active synthetic sample of pure (1*S*,3*S*,7*R*)-3-methyl-α-himachalene. In this study one of the (1*S**,3*S**,7*S**)- isomers of 3-methyl-α-himachalene co-eluted with the biologically active (1*S*,3*S*,7*R*)-3-methyl-α-himachalene. However, neither of the (1*S**,3*S**,7S*)-isomers of 3-methyl-α-himachalene occur in sex gland

Table 1
(a) Behavioural responses of *L. longipalpis* males and females to a hexane extract of the 3rd and 4th abdominal tergites of males in a wind tunnel. Control: 40–60 µl mixture of hexane and paraffin oil (1:1) on a filter paper disc

	Female			Male		
	Control	Pheromone extract		Control	Pheromone extract	
% Activation	31	68	**	37	83	**
% Attraction	0	25	**	8	55	**
No. insect tested	(35)	(44)		(38)	(42)	

(b) Behavioural responses of males and females *L. longipalpis* to (1*S*,3*S*,7*R*)-3-methyl-α-himachalene and to a synthetic mixture containing all eight isomers of 3-methyl-α-himachalene

	Female			Male		
	Control	1*S*,3*S*,7*R*-3-methyl-α-himachalene		Control	1*S*,3*S*,7*R*-3-methyl-α-himachalene	
% Activation	54	86	**	81	81	ns
% Attraction	0	31	**	12	45	**
No. insect tested	(35)	(36)		(42)	(42)	
	Control	Synthetic mixture		Control	Synthetic mixture	
% Activation	54	83	**	81	80	ns
% Attraction	0	33	**	12	33	*
No. insect tested	(35)	(30)		(42)	(30)	

(a): 4–6 male equivalents of the extract in 40–60 µl hexane/paraffin oil mixture (1:1) on a filter paper disc. Activation: flies that walked, hopped or flew less than 50 cm upwind. Attraction: flies that flew more than 50 cm upwind; *$p<0.05$, **$p<0.001$ compared to control, Fisher's exact test. Control: 40–60 µl mixture of hexane and paraffin oil (1:1) on a filter paper disc.
(b): 1–6 µg of (1*S*,3*S*,7*R*)-3-methyl-α-himachalene or the synthetic mixture in 40–60 µl hexane/paraffin oil mixture (1:1) on a filter paper disc. Activation: flies that walked, hopped or flew less than 50 cm upwind. Attraction: flies that flew more than 50 cm upwind; *$p<0.05$, **$p<0.001$ compared to control, ns = not significant, Fisher's exact test. Control as in (a).

extracts of *L. longipalpis* (Hooper et al., unpublished data) nor were they found to attract females in an earlier study (Hamilton et al., 1999).

Most remarkable is that both the naturally occurring and synthetic (1*S*,3*S*,7*R*)-3-methyl-α-himachalene evoked a response from the ascoid receptor cells that lasted for minutes. This apolar infochemical will easily adhere to the epicuticle of antennal structures and diffuse towards the pores of the ascoid sensillum over time, a phenomenon previously described for the bombykol receptor cells of *Bombyx mori* with (*Z,E*)-4,6-hexadecadiene (Kaissling et al., 1989). It must be assumed that the amounts delivered here to the electrophysiological preparation to evoke visible action potential bursts are far higher than what is required to induce physiologically relevant responses from ascoid receptor cells responsive to (1*S*,3*S*,7*R*)-3-methy-α-himachalene. The remarkable aspect is the lack of adaptation on the part of these receptor cells to continued stimulation.

Our behavioural studies support the electrophysiological data in that both male and female *L. longipalpis* flew upwind in the wind tunnel to a filter paper disk treated with either an extract of 4–6 male tergal gland equivalents, 1–6 µg of (1*S*,3*S*,7*R*)-3-methyl-α-himachalene, or the synthetic mixture of eight isomers of 3-methyl-α-himachalene. The synthetic mixture (1–6 µg) seems neither to improve nor to inhibit activation and attraction of *L. longipalpis* to (1*S*,3*S*,7*R*)-3-methyl-α-himachalene in the wind tunnel. Many insect semiochemicals are chiral compounds where the biological activity of each enantiomer differs, with the "unnatural" enantiomer (that not produced by the insect) being equally active, less active (but enhancing the activity of the natural isomer) or even inhibiting the activity of the active isomer (Mori, 1998). Our electrophysiological data suggest a high selectivity in the *L. longipalpis* olfactory system for the naturally occuring isomer produced by the males. Moreover, the fact that the synthetic mixture of 3-methyl-α-himachalenes containing unequal amounts of the eight isomers is as attractive as the pure (1*S*,3*S*,7*R*)-3-methyl-α-himachalene is quite fortuitous in relation to possible applications of pheromone mixtures for this disease vector in the field, considering the difficulties of enantioselective synthesis.

Kelly and Dye (1997) observed that the emigration rate of males from the lek was inversely related to host and fly abundance, and suggested that males were using semiochemicals to maintain aggregations; leks are maintained for many hours in *L. longipalpis* (Kelly and Dye, 1997). Quinnell and Dye (1994) showed in a field study of a domesticated population of *L. longipalpis* that males could attract not only females, but also other males in the presence of a host. Our laboratory study is in accordance

with this observation, indicating that (1*S*,3*S*,7*R*)-3-methyl-α-himachalene derived from male *L. longipalpis* can act as a sex attractant for females and also serves in inducing male responses. Male produced sex attractants often elicit both heterosexual and intrasexual responses resulting in aggregation, although the primary function of a male-produced pheromone is to attract a potential mate (Greenfield, 1980). Mate-finding systems based on male-produced sex attractants represent only 4% of known sexual attractants, and are not distributed among all insect orders (Landolt, 1997). Searching for a mate requires expenditure of considerable energy and greater exposure to predation as well as to unpredictable environmental conditions. Although attraction of other males is not beneficial for the emitter, it may evolve if the cost of "not calling" is larger than the cost of "having to share" the responding female (Wertheim et al., 2005). Being in group can facilitate mate-finding, resulting in energy saving. Lek formation in *L. longipalpis* often occurs on or near hosts (Dye et al., 1991; Quinnell and Dye, 1994). Male signaling in *L. longipalpis* may confer advantages to females other than just for mate finding, particularly when the signal is released at feeding and oviposition sites (Landolt, 1997).

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