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Behavioural and electrophysiological responses of the phlebotomine sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae) when exposed to canid host odour kairomones

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Abstract. Compounds from the odour-producing glands of the fox *Vulpes vulpes* were collected. This complex mixture of compounds was used to stimulate the 'ascoid' olfactory organs of female sandflies in single sensillum and gas chromatography-linked single sensillum recordings. Sixteen of these compounds were identified using gas chromatography-linked mass spectrometry and amounts present were determined. The compounds fell into four organic classes: ketones, carboxylic acids, alcohols and aldehydes. Specific neurones present in the ascoid sensillum that responded to each of these classes of compound were characterized.
A bioassay chamber was developed that gave female sandflies the choice of two odour sources. Female sandflies were attracted upwind by fox odour and were trapped in closer proximity to the fox odour port than the control port. Synthetic compounds were recombined in appropriate quantities to mimic the fox odour. In this bioassay, the synthetic blend attracted sandflies upwind, and again they were caught closer to the test port than the control port. Furthermore, the synthetic fox odour induced an electrophysiological response from neurones in the ascoid sensillum that was very similar to that induced by natural fox odour. No synthetic compound alone induced the same behavioural response from sandflies as did whole fox odour. However, benzaldehyde, 4-hydroxy-4-methyl-2-pentanone and 4-methyl-2-pentanone alone did cause sandflies to fly upwind and to be caught closer to the test port than the control.

Key words. Attractant, behaviour, electrophysiology, fox odour, *Lutzomyia longipalpis*, kairomones, odour bioassay, sandfly, sensillum

Introduction

The sandfly *Lutzomyia longipalpis* (Lutz & Neiva, 1912) exhibits opportunistic behaviour when seeking a host (Quinnell *et al*., 1992). It feeds on a range of hosts including dogs, cows, pigs, horses, birds, humans and opossums, with flies from different geographical locations showing different host preferences (Deane, 1956; Quinnell *et al*., 1992; Morrison *et al*., 1993). *Lutzomyia longipalpis* transmits the parasite *Leishmania chagasi*, and the disease, visceral leishmaniasis, has the greatest prevalence in rural areas of north-east Brazil (Vieira *et al*., 1990). The primary wild host of *L. longipalpis* and the sylvatic zoonotic reservoir is the South American fox *Cerdocyon thous* (L.) (Deane & Deane, 1954; Lainson *et al*., 1969). Infected dogs maintain the parasite in the peridomestic environment.

Lutzomyia longipalpis is a species complex with at least three sibling members (Ward *et al*., 1985; Lanzaro *et al*., 1993; Hamilton *et al*., 1996a,b). Males of each sibling population

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produce different terpenoid sex pheromones (Hamilton *et al*., 1994, 1996a,b) that form prezygotic mating barriers (Ward, 1986; Ward *et al*., 1989). Postzygtoic barriers cause certain mating crosses to reduce fecundity and alter sperm morphology (Ward *et al*., 1985; Lanzaro *et al*., 1993). Each sibling group produces a different intrapulse frequency of wing beats (Ward *et al*., 1988) and sound may also be an important species isolating mechanism at close range.

One factor that is known to affect the transmission rate of parasites is the number of bites a host receives (Lane, 1993). For man, this can be expressed as the level of anthropophagy of the vector. Ward *et al*. (1985) hypothesized that one member of the *L. longipalpis* species complex was a better vector of the *Leishmania* parasite, as its geographical distribution matched that of the endemic foci of the disease and it had a higher level of anthropophagy. Indeed, recent work has demonstrated differences in the Maxidilan gene of *L. longipalpis* from different geographical locations. Salivary gland products of this gene facilitate the entry of the parasite into macrophages, so enhancing the chance of infection in the host (Lanzaro *et al*., 1993).

Female sandflies endeavour to satisfy two requirements when they orientate towards their host: they acquire a blood meal to develop their eggs and are inseminated so that their eggs can be fertilized. The host and male sandflies each release semiochemicals which the female can perceive and orientate towards. The host releases kairomones, e.g. skin emanations (Hamilton & Ramsoondar, 1994), faecal contamination (Dougherty *et al*., 1995), carbon dioxide (Nigam & Ward, 1991) and possibly secretions from special glands, such as the uropygial gland of chickens (Nigam & Ward, 1991). The males lek and release terpene sex pheromones that attract conspecific females (Lane *et al*., 1984; Phillips *et al*., 1986; Hamilton & Ward, 1991; Hamilton *et al*., 1994). However, when present together, host odour and sex pheromone induce an additive interaction and the attraction of females is greatly enhanced (Morton & Ward, 1989a,b; Nigam & Ward, 1991; Hamilton, 1992).

Physical factors also play a role in the orientation of females to the blood meal host and these include temperature and humidity gradients (Nigam & Ward, 1991). *Lutzomyia longipalpis* has eyes with a spectral sensitivity similar to that of *Glossina morsitans* (Mellor *et al*., 1996), which are known to use vision in orientation towards a host. When sandflies choose a host, it is thought that size is important in the recognition process (Quinnell *et al*., 1992). Kelly & Dye (1997) showed that sandflies returned 'preferentially to the site of their previous night's aggregation', possibly indicating that sandflies can learn a 'familiar area map' using visual and olfactory cues.

Dougherty *et al*. (1995) took the first electrophysiological recordings from the ascoid sensillum (Boufana, 1990) on the antennae of female *L. longipalpis* and found neurones sensitive to the sex pheromone, oviposition pheromone (Dougherty & Hamilton, 1997) and compounds present in faecal material. These responses were characterized either by complex firing patterns of specific neurones, or by inhibition of other cells, followed by an increase in frequency when stimulation stopped.

In the present investigation, odours from the primary host of *L. longipalpis*, the fox, were used to stimulate the ascoid sensillum on the antennae of female sandflies to determine if they are perceived in this organ. Gas chromatography-linked single sensillum recording (GC-SSR) was used to identify compounds that the sandfly can perceive in fox odour. These compounds were then identified using gas chromatography-linked mass spectrometry (GC-MS) and tested by electrophysiological bioassay. The synthetic analogues were recombined in quantities present in fox odour and double-consecutive electrophysiology experiments were undertaken to determine if they reproduce the neuronal response induced by the natural host odour. A bioassay, which gave the fly a choice of two odour sources, was used to determine whether *L. longipalpis* was attracted to the fox odour. Synthetic analogues of compounds identified in fox odour were tested to determine whether they could induce a similar behavioural response to natural fox odour.

Materials and Methods

Sandflies

Lutzomyia longipalpis used in the investigation were originally collected from Jacobina, Bahia State, Brazil, and were reared over approximately 85 generations, according to the method of Modi & Tesh (1983). For the behavioural bioassay, females were isolated with an equal number of males for 5 to 6 days after emergence, at 27°C, 95% r.h. and LD 12 : 12 h. Female sandflies used for electrophysiology experiments were 2–3-days-old and were not blood-fed. All flies were allowed access to a saturated sucrose solution.

Biological extracts

The anal and caudal glands of five culled European foxes (*Vulpes vulpes*) were excised, placed into 50 ml ultra pure water (UHP UF, Elgstat, Elga, High Wycombe, U.K.), sealed under nitrogen in an air-tight bottle and stored at –70°C. Aqueous extracts were separated into 25-ml aliquots after an extraction period of 72 h. Each aliquot was mixed with 25 ml of dichloromethane (DCM) (all solvents used were pesticide residue analysis (BDH, Poole, U.K.) or ANALAR grade (Merck, Poole, UK). Samples were shaken vigorously for 5 min in a separating funnel, left for 30 min to settle and then drained off into their respective fractions. The DCM fraction was dried with a few grams of magnesium sulphate (Aldrich, Poole, UK) and reduced in volume to 2.5 ml in a fume hood at room temperature. Five samples of 500 µl were sealed in glass ampoules and stored at – 20°C. One 500-µl aliquot of extract was serially diluted to 10, 100, 1000 and 10 000 times dilution of the original sample.

Electrophysiology

Flies anaesthetized with CO_2 were mounted on double-sided sticky tape on top of a piece of Perspex set in Plasticine

(Dougherty *et al*., 1995). The sandfly was orientated at an angle of 20° to the horizontal, in a temperature- and humidity-controlled air stream (1 ms^{-1}, 100% r.h. and 26 ± 1°C). This ensured that the fly was exposed to a greater cross-section of the air stream. Another advantage of this arrangement was that condensed water near the preparation could drain away without affecting the recording. Recordings were made using tungsten electrodes, one placed into the scape of the sandfly antenna and the other at an angle of 120° into the base of the ascoid sensillum (antennal segments 3–5). Recorded signals were first preamplified (10^{12} Ω input impedance) and then passed into a AC/DC amplifier (UN-03, Syntech, Hilversum, The Netherlands), with the resultant signal being stored onto a digital audio tape recorder (DTR1200, Biologic, Grenoble, France). The signal was also played into a UN–03 interface box (Syntech) and then into an IDAC2 data acquisition card (Syntech) in an IBM-compatible 486 computer. This enabled signals from a single sensillum recording to be viewed and analysed using AUTOSPIKE (version 2, Syntech). Signals were simultaneously displayed on an oscilloscope (Tektronix, LR 37158, Marlow, U.K.) and hard copies of traces could be printed out using AUTOSPIKE software. Dougherty *et al*. (1995) previously used the criteria of spike amplitude, frequency and shape to discriminate different cell types in the multicellular recordings from the ascoid sensillum. The use of AUTOSPIKE meant that amplitude could be interpreted more accurately. Spikes were grouped together by their amplitude into classes (*X* axis), and plotted on a histogram against the number of spikes in each class (*Y* axis). By choosing individual classes it was then possible to analyse the reaction of specific groups of neurones to different chemical stimuli.

Electrophysiological stimulation

Stimulation was via a 5-ml polypropylene syringe, into which the stimulus was placed on a filter paper strip (Dougherty *et al*., 1995). A stimulus delivery system ST-05 (Syntech) was used to blow 2 ml of purified air for 1 s through the syringe and into the humidified air stream passing over the electrophysiological preparation (EP). For each stimulation the pre- and post-stimulus period was equal to the recorded stimulation period. To reveal specific cell types responding to chemical stimuli, 'double successive' stimulation was employed, in which the ascoid was stimulated with compound A for 1 s and then with compound B for 1 s.

The following chemicals, which were found to be electrophysiologically active by GC-SSR and identified using GC-MS, were used to stimulate the ascoid sensillum: 4-methyl-2-pentanone, 2-hexanone, 4-hydroxy-4-methyl-2-pentanone, 3-hydroxy-2-butanone, 4-methylheptanone, 2-butanol, 3-methylbutan-1-ol, 3-methyl-3-buten-1-ol, 1-pentanol, propanoic acid, 2-methyl propanoic acid, butanoic acid, 3-methyl butanoic acid, pentanoic acid, benzaldehyde and hexanal. All synthetic compounds were dissolved in hexane and were from commercial sources (>95% purity). For the electrophysiological bioassay the synthetics (100 µg/µl) were loaded (10 µl) onto a filter paper strip. After the solvent had evaporated, a drop of paraffin oil was placed onto the filter paper to reduce volatilization, and the filter paper was placed inside the syringe of the odour delivery system. The fox gland extract was used at 1, 10, 100, 1000 and 10 000 times dilution of the original sample. Once the quantity of the electrophysiologically active compounds present in 100 times diluted fox gland extract had been calculated it was recreated using synthetics at the correct dose and used to stimulate the ascoid sensillum. Compounds from different chemical classes were used in double-successive stimulations to determine whether different neurones in the ascoid sensillum were responding to them. The blend of synthetic fox odour was also used in double-successive stimulations with biological fox odour to see whether the synthetic compounds mimicked the natural products. Stimuli were tested on at least one ascoid sensillum of a minimum of ten flies.

Gas chromatography-linked single sensillum recordings (GC-SSR)

The GC (Carlo Erba 5160 Mega Series, Milan, Italy) was fitted with a fused silica capillary column (DBwax, J&W Scientific, Folsom, CA, U.S.A., 30 m × 0.25 mm i.d.; 0.25 µm film thickness), and was temperature programmed with an initial 6 min at 40°C, then a rise of 5°C min^{-1} to a final isothermal period (10 min) at 240°C. Carrier gas was hydrogen (1 ml/min) with on-column injection at 40°C and flame ionization detector (230°C). Before the flame ionisation detector (FID), the column eluent was split with a fused silica Y tube (split ratio 1 : 2), between the detector and the sandfly ascoid sensillum. The GC eluate passed via a heated transfer line (250°C), into the conditioned air stream. Any change in the overall sensitivity was tested by stimulating the ascoid before and after a recording with a known concentration of fox gland extract. The multicellular neuronal activity in an individual ascoid sensillum was separated from the background noise with a level discriminator built into the UN-03 amplifier. The action potentials were summated using a frequency-to-voltage converter (1 s time constant) and the subsequent DC signal was fed simultaneously with the DC output from the FID into the UN–03 interface box. The signal could then be observed in real time and later analysed to locate electrophysiologically active compounds using electroantennogram detection software (EAD; version 1.4, Syntech) on an IBM-compatible 486 computer. Any increase in the frequency of firing of neurones was visualized by a deflection in the DC signal in conjunction with a matching peak on the FID chromatogram. The AC signal was stored on the DAT for subsequent spike analysis. Hydrocarbon standards (n-alkane) were injected into the GC (C_{10}–C_{21}, 1 µl injection with 100 ng of each compound) and their retention times recorded to calculate Kovat's indices for the electrophysiologically active compounds in the fox gland extract. GC retention times are expressed as Kovat's indices relative to the retention time of n-alkanes.

Gas chromatography-linked mass spectrometry (GC-MS)

A Hewlett Packard GC-MS 5890 series II gas chromatograph (7971A mass selective detector; EI, 180°C, 70 eV) was used. Helium was the carrier gas under constant pressure (velocity 0.3 ms^{-1} at 40°C). The GC column and oven conditions were the same as for GC-SSR. Identification of a compound was performed first by matching its mass spectrum to that of a known product stored in the computer-based library of the GC-MS system. Identifications were confirmed by comparison of retention times (Kovat's indices) and mass spectra with synthetic compounds.

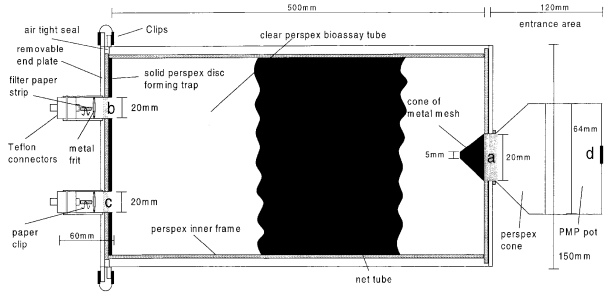

Fig. 1. Bioassay apparatus: b and c are the points of entry for the air flow into the bioassay tube, a is the exit point for the air flow and d is where the sandflies are introduced into the apparatus. For clarity the apparatus is not drawn to scale (see text for details).

Quantitative analysis of compounds in fox gland extract by gas chromatography

Gas chromatography (GC) was undertaken on 100-times diluted fox gland extract (the dilution that induced the greatest behavioural response from female sandflies). One microlitre of this sample was injected on-column at 40°C and five injections were performed. Analysis was performed on a column identical to GC-SSR experiments in a Shimadzu GC 17 A (Shimadzu, Milton Keynes, U.K.); the temperature programme was also the same as for GC-SSR and GC-MS experiments. The carrier gas (He) with an initial linear velocity of 1 ms^{-1} (at 40°C) was under constant pressure. The signal from the FID (230°C) was acquired using Shimadzu Class VP software (V4, Dyson Instruments). Calibration curves for synthetic compounds were calculated by injecting 1, 10, 100 and 1000 ng (four replicates each).

Host odour bioassay

The bioassay apparatus was constructed from a Perspex tube (Fig. 1) and Teflon tubing (1/4″) was used throughout. Inside the tube a frame was constructed from two Perspex discs (100 mm diameter) separated by three Perspex rod spacers (500 mm long). Fine nylon netting was stretched over this Perspex frame and over the rims of the Perspex discs to form a net cylinder running the length of the apparatus. The netting was used because sandflies have a hopping flight pattern and the material from which the flight chamber was constructed could have affected the movement of the insects (Morton &

Table 1. Retention times (Rt.) for GC-MS and Kovat's indices (K_i) of the sixteen compounds present in an extract of fox-gland volatiles eliciting electrophysiological responses from neurones in the ascoid sensillum on the antennae of female *L. longipalpis*, quantities of these compounds in 100-times diluted fox gland extract, and the number of times each compound induced an electrophysiological response in the twelve GC-SSR analyses.

Identified compound	Rt. GC-MS (min)	K_i GC-MS	K_i GC-SSR	No. of observed stimulations in GC-SSR	Quantity ng/µl of 100× diluted extract
4-Methyl-2-pentanone	7.36			5\12	28
2-Hexanone	8.54	1026	1024	4\12	10
4-Hydroxy-4-methyl-2-pentanone	18.81	1314	1312	7\12	328
3-Hydroxy-2-butanone	16.29	1227	1226	8\12	52
4-Methylheptanone	25.06	1540	1538	3\12	16
2 Butanol	8.95	1041	1039	9\12	58
3-Methylbutan-1-ol	14.17	1172	1169	7\12	102
3-Methyl-3-buten-1-ol	15.38	1199	1201	4\12	13
1-Pentanol	15.52	1203	1205	8\12	33
Propanoic acid	23.69	1484	1481	8\12	59
2-Methyl-propanoic acid	24.26	1511	1509	8\12	152
Butanoic acid	25.65	1562	1559	7\12	226
3-Methyl butanoic acid	27.63	1645	1641	2\12	318
Pentanoic acid	27.33	1631	1628	7\12	57
Benzaldehyde	22.86	1452	1450	8\12	36
Hexanal	9.90	1079	1076	6\12	28

Ward, 1989a,b). A removable end-plate had a circular, air-tight foam seal which pressed against the Perspex bioassay tube and was fastened with Delrin keck clips (Aldrich, Poole, U.K.). The other end had an entrance area (120 mm × 64 mm), formed from a machined Perspex cone which fitted onto a 125-ml polymethylpentene pot (Nalgene, BDH). The flies were introduced here through a netting-covered hole (point d, Fig. 1). The sandfly entrance to the bioassay tube was a 20-mm diameter hole (point a, Fig. 1) and using a cone of fine metal mesh, with a 5-mm port at the tip prevented re-entry of the flies to the release chamber. To create an air-flow through the apparatus, bottled, zero grade air (BOC, Guildford, U.K.) was fed through two charcoal traps (Alltech, Carnforth, U.K.) placed in series into a CD-02/E stimulus controller (Syntech) that gave a constant flow of 0.1 ms^{-1} (Airflow meter, KM4007, Comark Ltd, Welwyn Garden City, U.K.) at air entry ports b and c (20 mm diameter). The air was humidified by bubbling it through two gas-wash bottles maintained in a heated water bath (Tempette, TE-8 J, Techne, Duxford, U.K.). The single air-line was then split, using a Teflon T-piece, and two arms of equal length were taken to Teflon connectors in ports b and c. This system allowed the temperature and humidity to be controlled independently of the insectary in which the experiments were conducted. The mean air temperature and humidity (Digital Thermos-Hygrometer, RS 213-802, RS, Stockport, U.K.) emanating from ports b and c was 28.1–77 ± 0.85°C and 95–77 ± 3.2% r.h., whilst the air at port d was, 27.5 ± 0.89°C 65 ± 3.9% r.h. Ports b and c contained a metal frit to prevent access by the flies and to maintain a smooth air flow. This was confirmed visually when ammonium chloride vapour was passed through the connectors and maintained two discrete cones until reaching a point 100–120 mm down the tube where mixing occurred.

The air stream was switched on and allowed 10 min to stabilize, after which the temperature, humidity and air-flow were measured and recorded for every replicate. A filter paper strip (10 mm × 5 mm) with the test sample was placed in the test port (b or c) and a filter paper with solvent only in the other. The test ports were alternated between replicates and the side containing the test filter paper was recorded. Approximately 30 s later ten flies were introduced into the entrance chamber. As the flies normally feed after sunset, they were

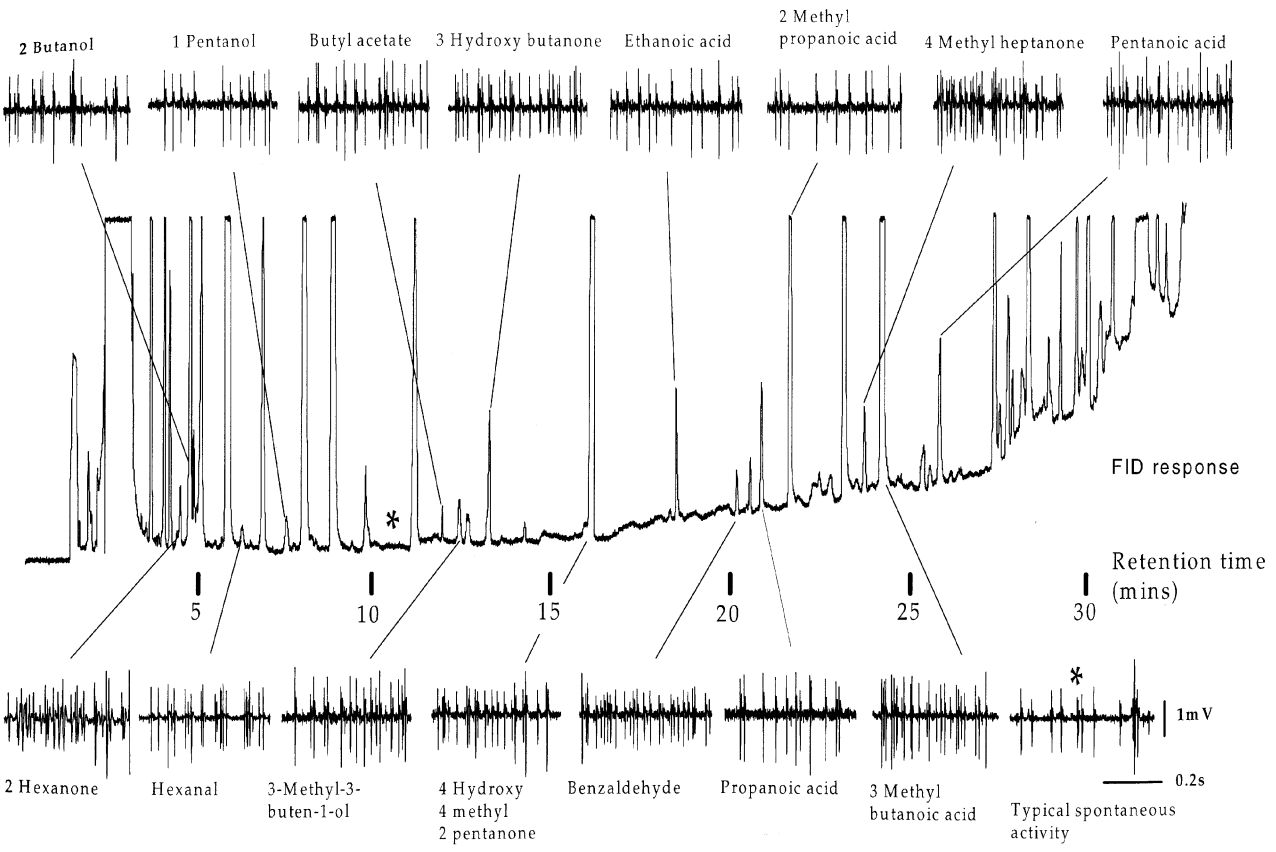

Fig. 2. Analysis of fox gland volatiles by gas chromatography-coupled electrophysiological recording from an ascoid sensillum of a female *L. longipalpis*. The central trace is the flame ionization detector (FID) response. The spike trains above and below the FID trace show the action potentials that were generated by neurones in the ascoid sensillum. The line from each spike train to the FID trace identifies the compound being delivered to the ascoid when the increase in the action potential frequency was recorded onto the digital audio tape; * denotes typical spontaneous activity. The names of the individual chemostimulants, identified by GC-MS and GC (Table 1), are shown above or below the spike trains.

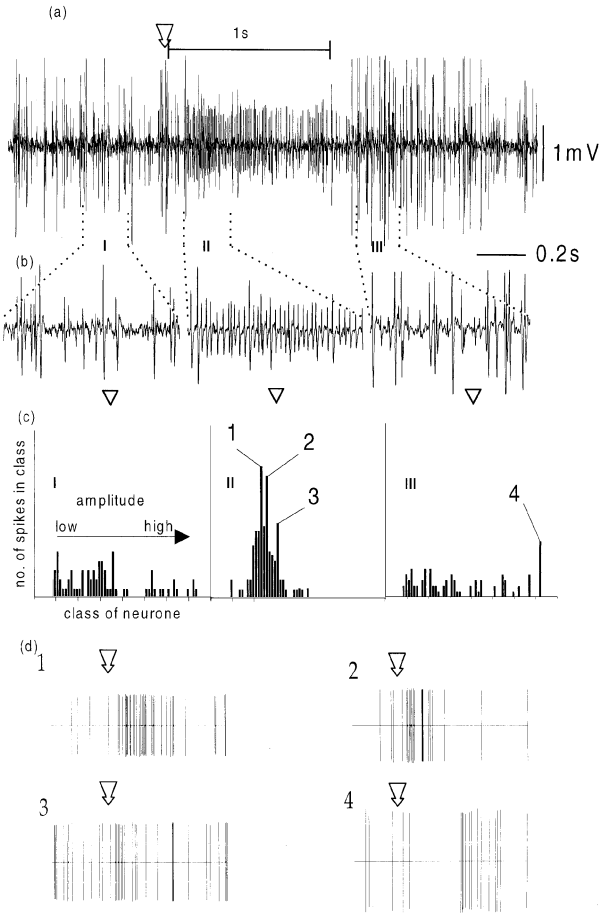

Fig. 3. (a) Electrophysiological response of olfactory cells within the ascoid sensillum on the antenna of a female *L. Longipalpis* to 10-times diluted extract of fox gland volatiles; the open arrows denote onset of stimulation for 1 s; (b) expansion of regions before (I), during (II), and after (III) stimulation; (c) histograms of spike frequency against spike amplitude classes for periods I, II and III; (d) response patterns during and post stimulation of each of the four neurones classified according to amplitude in (c) as extracted from the recording in (a).

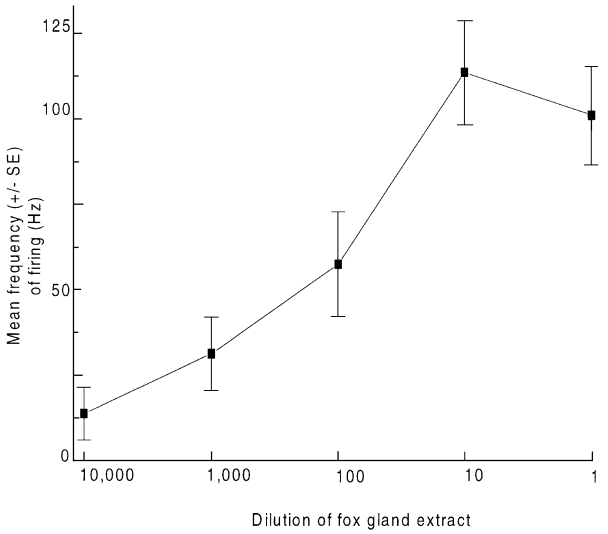

Fig. 4. Dose–response curve (mean frequency ± SE of all neurones firing) for electrophysiological responses of the ascoid sensillum receptors on the antennae of female *L. longipalpis* ($n = 10$) to an extract of fox-gland volatiles.

conditioned in the dark for 2 h before the experiment. The experiment was set up in low light generated by a cold light source with red filters attached (BDH). After introducing the flies, the lamp was switched off and they were given 5 min to respond to the odour. A second Perspex disc (trap disk), with two holes that matched the size and position of ports b and c, was placed against the end-plate. This trap disc was coated with castor oil (Thornton and Ross, Huddersfield, U.K.), which previously has been used successfully to catch sandflies in unbaited traps in the field (Cameron *et al*., 1995a,b).

The test samples were loaded onto a filter paper strip in 2–5 µl hexane, which was held in place with a paper clip. Synthetic compounds and mixtures were bioassayed at the quantities found in 100-times diluted fox gland extract (Table 1). Each test or control bioassay was replicated ten times. Between each experiment the apparatus was washed with distilled water, methanol and distilled water once more, and then dried at room temperature. The net tubes running the length of the bioassay apparatus were replaced between each replicate. They were washed using Teepol (Orme Scientific, Manchester, U.K.), rinsed thoroughly in distilled, deionized water and allowed to dry at room temperature before re-use.

Statistical analysis

For each test sample or control, the number of flies caught in the castor oil at the end of the 5-min assay was recorded, and any difference in the number of flies caught between the control and the test samples was analysed using χ^2. To establish whether the flies showed a preference for one of the delivery ports the distance of each trapped fly from the centre of the test port (x_t) and the control port (x_c) was recorded and the means calculated. This was done by laying the Perspex trap disc, with the flies still in place, onto a piece of acetate with a pre-drawn template of the plate to line up the correct orientation. The position of the flies was then marked onto the acetate and the castor oil removed. The position of the flies on the stencil was then transferred onto a sheet of paper and scanned using a flat-bed A4 scanner (Deskscan Colour, Primax, Abingdon, U.K.) into Finishing Touch (v1.1, Ulead Systems Inc, Torrance, CA, U.S.A.) image handling software. Images were scanned using the same scale and resolution parameters. Each image was then transferred as a TIF file into Micrographx Designer (v3.1, Micrographx Inc, Richardson, TX, U.S.A.), and in this form a line could be drawn between the mark representing a fly and the centre of each port. The

length of these lines was then calculated by the software to the nearest 0.1 mm. The difference in distance (x_d) of each fly, between the centre of the test port and the control port was calculated ($x_d = x_c - x_t$). The data were then transferred into SPSS (v6.1, SPSS Inc, Woking, U.K.) and Minitab (v10.2, Minitab, Coventry, U.S.A.) for data analysis. If no bias towards a single port existed, then $x_d = 0$. Alternatively, if a bias towards a particular port existed then $x_d \neq 0$. As fox odours were expected to be attractive a one-tailed, paired *T*-test was undertaken on x_d to determine if it was significantly different from zero; if bias towards the test port existed then $x_d > 0$. To investigate possible differences of x_t between treatments bioassayed, the data were log-transformed and ANOVA followed by Tukey's multiple comparison test was performed. A block design was used to take into account variance due to which side of the bioassay chamber the test sample was placed. To analyse the distribution of flies around the test port, the Kolmogorov–Smirnov goodness-of-fit test was undertaken on x_t, showing whether the sandflies caught around the test port had a normal or uniform distribution for each of the treatments.

Results

Up to sixteen compounds in the fox gland extract elicited electrophysiological responses from the ascoid sensillum on the antennae of female sandflies (Fig. 2) during twelve replicate analyses. These were identified by GC-MS and their retention times were compared with those of synthetic standards. The compounds are shown in Table 1 with retention data and quantities present.

Electrophysiological responses to fox gland extract

The responses from neurones within the ascoid sensillum to fox odour were complex and multicellular (Fig. 3a,b). The histogram of amplitude classes versus number of neurones in each class (Fig. 3c) allowed the selection of the specific

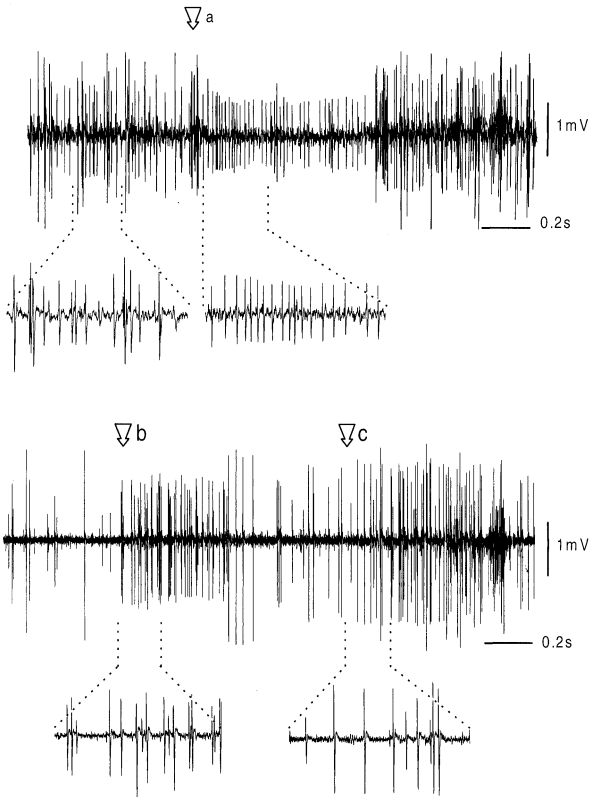

Fig. 5. Electrophysiological responses of olfactory cells within the ascoid sensillum on the antenna of a female *L. longipalpis* to (a) a combined mixture of synthetic ascoid chemostimulants that mimic levels present in 100-times dilute fox gland odour, and consecutive stimulation with (b) benzaldehyde and (c) 3-methyl-butanoic acid. Open arrows on the upper traces in (a), (b) and (c) indicate onset of stimulation for 1 s; the lower traces are expanded sections of the upper traces.

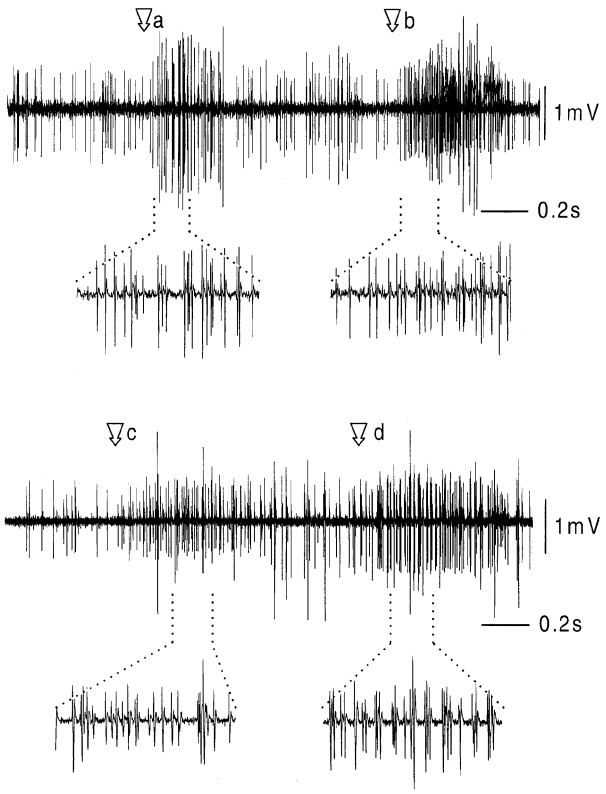

Fig. 6. Electrophysiological responses of olfactory cells within the ascoid sensillum on the antenna of a female *L. longipalpis* to stimulation with (a) butanoic acid followed by (b) 3-methyl-3-buten-1-ol, and (c) 4-hydroxy-4-methyl-2-pentanone followed by (d) butanol. Open arrows on the upper traces indicate onset of stimulation for 1 s; the lower traces are expanded sections of the upper traces.

Table 2. The behavioural responses of female *L. longipalpis* in the host odour bioassay to fox-gland extract, individual synthetics, combined synthetics and a solvent control. x_t and x_c are the distances of each fly from the centre of the test and control ports, respectively. In Tukey's multiple comparison test on the log of X_d ($X_c - X_t$), treatments with the same letter were not significantly different in their ability to bias female *L. longipalpis* towards the test port. In the Kolmogorov–Smirnov test on x_t if $P < 0.05$ for either U or N, then the flies were not uniformly and/or normally distributed around the test port. The number of flies caught on the trap disc of the bioassay apparatus for each test treatment was compared to the control using χ^2: the asterix signifies a test treatment that attracted significantly more ($P < 0.01$) female *L. longipalpis*.

Odour source	Mean distance (mm ± SE) from (χ^2) x_t	x_c	Tukey's test on log x_t	Kolmogorov -Smirnov of $x_t(P)$ U	N	Mean no. of flies caught (± SE)	*P*
Fox odour dilution							
10	439.1 ± 20.3	313.5 ± 16.7	D	0.4	0.914	4.2 ± 0.32	0.02
100	205.7 ± 9.5	467.67 ± 9.69	A	0.001	0.003	9.9 ± 0.10	0.001*
1000	287.0 ± 12.9	423.3 ± 16.9	B	0.068	0.069	6.7 ± 0.39	0.001*
10 000	386.5 ± 38.3	366.9 ± 27.4	CD	0.931	0.978	1.7 ± 0.37	0.552
Control	334.9 ± 29.2	357.1 ± 21.0	C	0.959	0.782	2.1 ± 0.31	
Combined synthetics	223.7 ± 8.92	467.0 ± 11.8	A	0.001	0.004	9.9 ± 0.10	0.001*
4-Methyl-2-pentanone	247.5 ± 15.2	425 ± 13.2	AB	0.009	0.011	8.6 ± 0.12	0.001*
4-Hydroxy-4-methyl- 2-pentanone	263.4 ± 18.2	446.4 ± 20.1	AB	0.001	0.009	9.6 ± 0.22	0.001*
2-Butanol	378.4 ± 29.9	385.5 ± 28.7	CD	0.845	0.894	2.7 ± 0.34	0.437
3-Methylbutan-1-ol	404.9 ± 29.6	372.2 ± 32.8	CD	0.855	0.934	1.7 ± 0.37	0.552
1-Pentanol	389.2 ± 32.2	365.3 ± 26.5	C	0.956	0.952	2.0 ± 0.35	0.687
Propanoic acid	371.5 ± 18.5	361.0 ± 22.1	C	0.790	0.854	9.5 ± 0.36	0.001*
2-Methyl propanoic acid	374.2 ± 24.3	385.3 ± 19.8	CD	0.894	0.899	9.2 ± 0.39	0.001*
Butanoic acid	368.8 ± 20.2	362.3 ± 14.4	CD	0.912	0.954	9.1 ± 0.35	0.001*
3-Methyl butanoic acid	356.6 ± 25.6	349.5 ± 19.4	CD	0.895	0.913	9.0 ± 0.39	0.001*
Benzaldehyde	300.1 ± 15.8	437.6 ± 18.1	B	0.001	0.002	6.2 ± 0.33	0.001*
Hexanal	338.7 ± 19.5	355.8 ± 19.2 C	C	0.311	0.627	3.8 ± 0.31	0.052

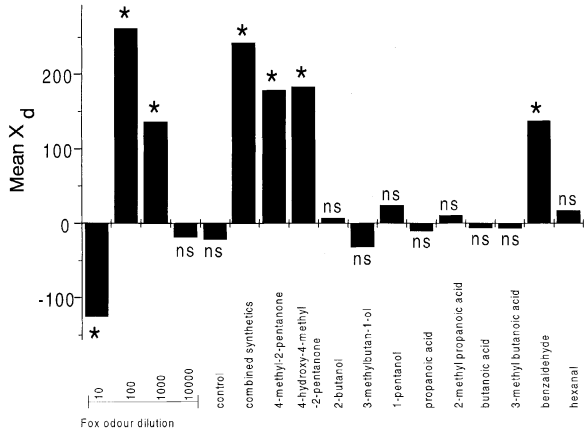

Fig. 7. Bias of trapped female *L. longipalpis* towards test ($x_d > 0$) and control ($x_d < 0$) ports of an olfactometer (see text) in which an extract of fox gland volatiles, individual synthetic *L. longipalpis* olfactory chemostimulants and the combined synthetic chemostimulants were tested at levels present in 100-times diluted fox gland extract, and a solvent control. * denotes a treatment that significantly (one-tailed paired *t*-test; $P < 0.001$) biased female sandfly responses; ns = not significant.

neurone types that responded to fox gland odour. Using the amplitude discrimination histogram of AUTOSPIKE and visual comparison, it was possible to discern at least three classes of neurone that increased their frequency of firing during stimulation with the fox gland extract. One cell (Fig. 3d cell 1) increased in frequency throughout the period of stimulation, whilst the other two cells (Fig. 3d cells 2 and 3) increased in frequency only briefly during the first period of stimulation. The response was characterized by inhibition of the largest amplitude action potentials. When stimulation stopped, these neurones responded by increasing frequency above their resting frequency (Fig. 3d cell 4). The response to fox gland extract was dose-dependant (Fig. 4), and the highest activity was observed (all responding cells counted) to ten-times diluted fox gland extract.

Electrophysiological responses to synthetic compounds

All electrophysiologically-active compounds isolated by GC-SSR stimulated neurones in the ascoid sensillum when presented at a dose of 1 mg in the syringe of the odour delivery system. A blend of synthetic fox gland volatiles induced an electrophysiological response in the ascoid sensillum similar to the biological extract of the same concentration (Fig. 5a). The aldehydes, hexanal and benzaldehyde, activated the same cell, with a high amplitude spike characterized by relatively equal positive and negative components (Fig. 5b), a response similar to that observed from cell 3 when stimulated with fox odour (Fig. 3). Similar to the response induced by fox odour, benzaldehyde caused inhibition of cells producing high-

amplitude action potentials (Fig. 5). The alcohols, 2-butanol, 3-methylbutan-1-ol, 3-methyl-3-buten-1-ol and 1-pentanol, caused a second cell, which was different from the aldehyde-sensitive cell, to increase firing frequency. This cell was characterized by action potentials that were lower in amplitude than those produced by the hexanal/benzaldehyde-sensitive cell (examples in Fig. 6). The response of the alcohol-sensitive cell was similar to that observed for cell 2 when exposed to fox odour (Fig. 3). The ketones, 4-methyl-2-pentanone, 2-hexanone, 4-hydroxy-4-methyl-2-pentanone, 3-hydroxy-2-butanone, 4-methylheptanone, stimulated a third type of cell (example in Fig. 6) characterized by action potentials with an amplitude lower than those of the neurones sensitive to either the aldehydes or alcohols. Ketone-sensitive cells showed a response similar to that observed from cell 1 when exposed to fox odour (Fig. 3). The carboxylic acids, propanoic, 2-methyl propanoic, butanoic, 3-methyl butanoic and pentanoic, all stimulated a neurone with a high amplitude, although not the cell with the highest spike amplitude observed (examples in Figs 5 and 6). This acid-sensitive neurone was not involved in the response to the whole fox gland extract, nor in the response to the synthetic mixture that mimics the fox gland blend, but was possibly one of the cells which were inhibited and which returned with a rebound when stimulation stopped (Figs 3 and 5). Neither inhibition nor rebound firing was observed following stimulation with the acids on their own. Double-successive stimulations using the synthetic compounds showed that different neurones were responding to each of the four classes of compound with different functional groups (Figs 5 and 6). Stimulation with air, solvents or paraffin oil caused no significant increase in spike frequency of any of the neurones.

Behavioural responses of female sandflies

Table 2 shows x_t and x_c data for the natural and synthetic compounds tested. The extract of fox gland (100- and 1000-times dilution), combined mixture of synthetic olfactory chemostimulants (equivalent of 100-times dilute fox gland extract), benzaldehyde, 4-hydroxy-4-methyl-2-pentanone and 4-methyl-2-pentanone induced a significant bias, with females being preferentially caught closer to the test port than the control. With the exception of the response to the mixture of synthetics, this bias is represented by a value of x_d that is significantly greater than zero (Fig. 7). The biasing effect was lost when 10 000-times diluted gland extract was used. By contrast the 10-times diluted fox gland extract induced a significant bias away from the test port and was the only sample where this was observed (Fig. 7). No other synthetic compound or solvent control induced a bias in the distribution of the females on the trap disc. The measurement x_t was not normally or uniformly distributed for the treatments that biased the flies towards the test port (Table 2), therefore the data were log-transformed. The ANOVA showed that there was highly significant variation between samples ($P < 0.001$), but there was no significant variation due to the side of the bioassay on which the test sample was placed ($P > 0.05$). The Tukey's multiple comparison test (Table 2) showed that 100-times diluted fox gland extract, combined synthetics, 4-methyl-2-pentanone and 4-hydroxy-4-methyl-2-pentanone trapped female flies closer to the test port than all the other biological extracts and synthetic compounds tested. The 1000-times diluted fox gland odour and benzaldehyde trapped the flies closer to the test port than the fatty acids and alcohols, which in turn were not significantly different from the control. One hundred- and 1000-times diluted fox gland odour, combined synthetics, benzaldehyde, all the ketones and carboxylic acids tested caught statistically more flies in the castor oil on the trap disc than the solvent control. However, the alcohols and hexanal failed to trap more flies than the control.

Discussion

Receptor neurones in the ascoid sensillum on the antennae of female *L. longipalpis* responded in a dose-dependant manner to extracts of fox gland odour. Sixteen constituents, representing ketone, alcohol, aldehyde and carboxylic acid constituents of the gland extract, were identified as olfactory stimulants. The carboxylic acid cell produced a high amplitude action potential, although not the highest present in the spike train. The neurone sensitive to aldehydes produced a lower-amplitude action potential, which appeared to inhibit the carboxylic acid and other neurones producing high-amplitude spikes. After stimulation with the aldehydes there was a 'rebound', i.e. an increase in frequency above the normal spontaneous rate of the inhibited neurones. Alcohols stimulated a third cell, which produced an intermediate amplitude action potential, and caused no inhibition. Ketones stimulated a fourth neurone with a low-amplitude action potential. The responses to these compounds were complex, but the amplitude classification and extracted spike trains using AUTOSPIKE combined with double successive stimulations strongly suggested that separate cells were responding to the four classes of compound. The selectivity of these neurones to specific compounds within these classes is unknown and further work is required to elucidate this. The synthetic compounds identified as electrophysiologically active by GC-SSR were reconstituted in a blend, matching levels present in the electrophysiologically and behaviourally active fox gland extract. The electrophysiological response to this synthetic mixture matched that induced by the biological extract.

The 100-times dilute fox gland extract attracted flies up the bioassay chamber, trapped significantly more flies than the control and caused a bias, with female flies being trapped in a non-normal or uniform distribution closer to the test port than the control. This induced behaviour was mimicked by the recombined blend of synthetic chemostimulants, at levels present in 100-times diluted fox gland extract, which evoked a clear electrophysiological response from the ascoid. The ketones and benzaldehyde alone also induced this behaviour, although the response was not as clear-cut as with the biological extracts. The carboxylic acids induced more flies to be trapped than the control, but did not alter the distribution

of the flies, with no apparent bias towards or away from the test port being recorded, and the alcohols induced no observable behavioural response in the bioassay.

Dougherty *et al*. (1995) identified the neurones that were sensitive to aldehydes and alcohols in host faeces, and the action potentials and inhibitions induced match those described here. Hexanal and 2-methyl-2-butanol were shown to reproduce the oviposition response induced by vertebrate faecal extract. It is surprising that neither hexanal nor the methyl-branched alcohol tested here induced a behavioural response from the flies. However, the flies tested here were not gravid. The oviposition pheromone of *L. longipalpis* was found to be dodecanoic acid, and a neurone sensitive to this compound was located in the ascoid sensillum (Dougherty & Hamilton, 1997). This compound alone induced the oviposition behavioural response normally caused by a hexane extract of sandfly eggs and, furthermore, acted synergistically with hexanal and 2-methyl-2-butanol to give a greatly enhanced and targeted oviposition response (Dougherty & Hamilton, 1997).

Lutzomyia longipalpis has catholic feeding tendencies (Quinnell *et al*., 1992) and this phenomenon is reflected in its ability to perceive a wide range of compounds that emanate from hosts. The ascoid sensilla of *L. longipalpis* can house between three and eleven olfactory receptor cells and other olfactory sensilla also occur on the antenna (M. Vlimant and P. A. Diehl, unpublished data). The dried fruit beetle, *Carpophilus hemipterus* (L), is a generalist herbivore and is known to respond in a wind tunnel to eighteen chemical components of banana odour (Phelan & Lin, 1991). The simplest blend that induced a full behavioural response contained at least four compounds representing three functional groups. These compounds could be exchanged, in the active blend, by similar products with the same functional group and the full behavioural response was maintained. The authors concluded that the 'generalist insect herbivore locates its hosts by a long-range response to a variety of blends of common fruit volatiles'. *Lutzomyia longipalpis* may similarly be able to perceive and orientate towards a variety of different hosts. Furthermore, it may be possible that different members of the *L. longipalpis* complex are attracted to different hosts due to subtle differences in their sensory system.

Benzaldehyde, 4-methyl-2-pentanone and 4-hydroxy-4-methyl-2-pentanone tested alone almost matched the full behavioural response induced by whole fox gland extract, therefore further work is required to mimic the simplest blend that induces this response. When an insect perceives a complex odour, and no single compound induces a full behavioural response, it is thought they use a large number of neurones with sensitivities that overlap (Isman, 1992). Generally the transfer of this complex information to the brain is via 'across-fibre patterning'. This allows the development of a search template and success may lead to strengthening of a behavioural response next time the stimulus is encountered. It is known that the parasitoid *Microplitis croceipes* uses visual and olfactory cues in learning to concentrate its search patterns for plant structures that are most profitable in terms of host encounters (Wacker & Lewis, 1994). Furthermore, the combined effect of these two modalities on learnt behaviour of the parasitoid was additive. When *L. longipalpis* orientates towards a host it is attracted by means of host odour, sex pheromone and possibly other sensory modalities, such as vision (Quinnell *et al*., 1992). At the host, once the sandfly satisfies the requirements of insemination and a blood-meal, the search template is strengthened and adaptive learning takes place. This could explain why Kelly & Dye (1997) observed sandflies returning to the lekking site at which they had been observed the previous night. Learning experiments are required to investigate this mechanism, as a female's ability to recognize a 'learnt' host has implications for the transmission model of *Leishmania*.

An important kairomone that is known to attract haematophagus insects is carbon dioxide (CO_2) (Takken, 1991) and for *Aedes aegypti* it must be present with other more specific host odours before attraction can be induced (Smith *et al*., 1970; Price *et al*., 1979). *Anopheles gambiae* is highly anthropophillic and is thought not to use CO_2 as its primary host-locating kairomone, but rather to employ more specific fatty acid compounds produced by bacteria present on human skin (Knols, 1996). If one accepts the hypothesis that one member of the *L. longipalpis* species complex is more anthropophillic than others (Ward *et al*., 1985, 1988; Hamilton & Ramsoondar, 1994), and is hence a better vector, then the role of CO_2 and its interaction with the compounds identified here must be investigated. This may reveal kairomone specificity and show up differences in potential host range between members of the sibling complex. It is also important to note that the humidity and temperature gradients are known to be important in the orientation of some haematophagus insects (Lehane, 1991). However, in the present investigation the different responses from flies exposed to fox gland odour and the control with a 30% r.h. drop along its length would tend to indicate that sandflies use olfaction primarily in their host orientation responses.

The alcohols that induced an electrophysiological response from the ascoid sensillum, but failed to invoke a behavioural response, are present in the odour plume of plants (Knudsen *et al*., 1993). Sandflies are known to obtain sugar meals from plants, and *L. longipalpis* has been shown to have associations with specific plants (Cameron *et al*., 1994) and their colonizing aphid species in Brazil (Cameron *et al*., 1995a). In a different physiological state, with the females undertaking a search for a sugar meal, these plant-associated alcohols may play an important role in the orientation of the sandfly to plants. The ascoid on the antennae of *L. longipalpis* is also known to contain receptors sensitive to the terpenes such as pinene isomers and α-terpinene (Dougherty *et al*., 1995).

The chemical constituents of fox odour are known to include ketones, aldehydes, fatty acids, alcohols and sulphide compounds (Albone *et al*., 1974; Bailey *et al*., 1980), and these compounds are thought to play a role in individual and group recognition, territorial marking, markers in food scavenging, sexual recognition, indicators of reproductive status and possible pheromone function (Jorgenson *et al*., 1978). *Phlebotomus papatasi* is known to be attracted to dimethyl disulphide (Wilson *et al*., 1989a,b, 1990), one of the compounds that causes the unpleasant smell of fox odour. However, Dougherty

et al. (1995) found no electrophysiological or behavioural response when *L. longipalpis* was exposed to this product, nor was any response noted in this study. The difference between the two species of sandfly may indicate adaptations to different host ranges.

When the sex pheromone of *L. longipalpis* is presented with host odour to virgin females, a synergistic effect is observed on attraction (Morton & Ward, 1989b; Hamilton, 1992). With the characterization of some of the host odour kairomones which the sandfly uses to locate a blood-meal host, this interaction may now be studied in more detail. Present research is investigating the interaction of the sex pheromone with blends of synthetic host odour components to reveal the nature of the synergism at an electrophysiological and behavioural level. Insight into this interaction, with development of specific blends for different members of the *L. longipalpis* complex, is seen as a prerequisite to the development of sibling-specific, semiochemical-baited, monitoring traps.

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