

Identification of oviposition attractants for the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae) in volatiles of faeces from vertebrates

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Abstract. Extracts of volatiles from rabbit and chicken faeces preferentially attracted gravid sandflies, *Lutzomyia longipalpis* (Lutz and Neiva), in an oviposition bioassay. In electrophysiology experiments, the same extracts selectively stimulated two olfactory cells while inhibiting another in ascoid sensilla on the antennae of these flies. Analysis of faeces volatiles by gas chromatography linked to ascoid sensillum recording revealed two early eluting electrophysiologically active components of rabbit faeces. These active compounds were identified in both rabbit and chicken faeces volatile extracts by gas chromatography-mass spectrometry as hexanal and 2-methyl-2-butanol. Hexanal stimulated one cell type and inhibited another, whereas 2-methyl-2-butanol stimulated a third cell type. A 1:1 mixture of synthetic hexanal and 2-methyl-2-butanol elicited the same targeted oviposition response from gravid females on the treatment septum of the bioassay as did the total volatile extract of rabbit or chicken faeces.

The monoterpenes $\alpha(+)$ -pinene (plus some optical and positional isomers) and α -terpinene activated a separate cell type, whereas benzaldehyde stimulated the same receptor as hexanal, but with a higher threshold. Furthermore, an olfactory cell selectively tuned to the perception of the male sex pheromone of this species was also found in the ascoid sensillum.

Key words. *Lutzomyia longipalpis*, Phlebotominae, oviposition, attraction, hexanal, 2-methyl-2-butanol, olfactory sensillum, electrophysiology, gas chromatography coupled single sensillum recordings.

Introduction

The sandfly *Lutzomyia longipalpis* (Lutz & Neiva) is the vector of visceral leishmaniasis in Latin America where 1.6 million people are considered to be at risk of infection, and over 16,000 cases occur annually (Ashford *et al.*, 1992). Sandflies are known to lay up to 100 eggs in a single oviposition and pass through several gonotrophic cycles (Dye *et al.*, 1987). Females lay their eggs in microhabitats such as cracks and crevices of chicken coops and cow pens, which are rich in organic nutrients (Ward, 1974). The eggs are laid under conditions of stable humidity (Bettini & Mellis, 1988), and it is known that optimal

temperature and humidity are important influences on oviposition (ElNaiem, 1991). The presence of a thigmotropic response from gravid sandflies was demonstrated in the laboratory, with females laying eggs preferentially in crevices and folded paper strips (ElNaiem & Ward, 1992a).

ElNaiem & Ward (1992b) showed that phlebotomine colony frass, larval rearing medium and rabbit faeces contained semiochemicals that served as oviposition attractants and stimulants for *L. longipalpis*. Furthermore, an oviposition attractant and stimulant also existed in standard rabbit food and hay. Moreover, when an extract of rabbit food was applied in combination with an oviposition pheromone associated with the eggs (Dougherty *et al.*, 1992), the behavioural response was highly targeted on the test substrate (Dougherty *et al.*, 1993). This phenomenon was used to develop a laboratory trap incorporating the combined semiochemicals which caught on

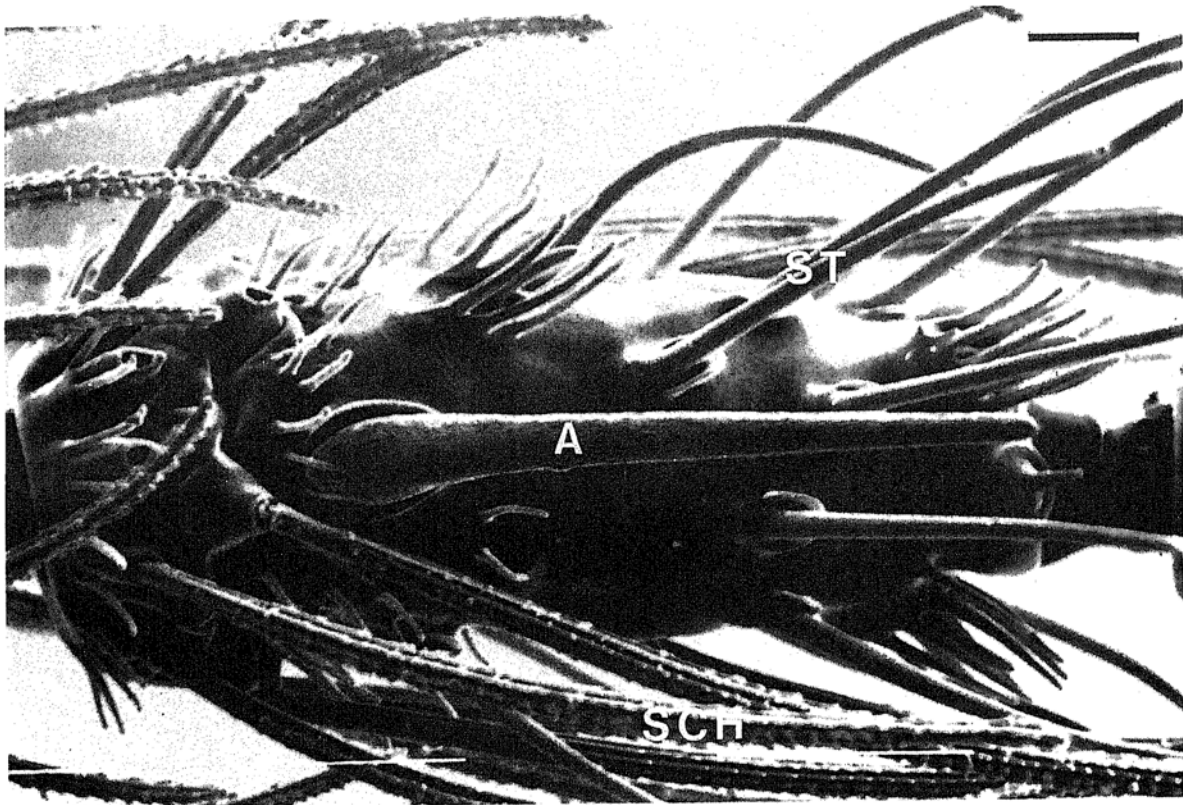


Fig. 1. Scanning electronmicrograph of the ascoïd sensillum (A) on antennal segment 14 of a male *L. longipalpis*. Ascoids are set proximally on the lateral face of segments 3–15; the ascoid is approximately the same length as the segment here. The bar (upper right) is 5 μm . Other large sensilla include SCH = sensillum chaeticum and ST = sensillum trichodeum (after Boufana, 1990).

average 62% of exposed gravid females over a 72 h period.

Volatile semiochemicals could be perceived via olfactory sensilla on the antennae of *L. longipalpis*, which bear a range of setae (Fig. 1). The most likely location for receptors is within the large walled-pored ascoïd sensillum (Boufana, 1990). Ascoids are paired structures borne laterally on antennal segments 3–15 of both sexes. They are transparent sensilla with many pores in the sensillum wall. The ascoids of males are longer than those of females, and variations in ascoid length and distribution have been used for taxonomic purposes. The ascoids of females are more highly innervated than those of males. Although ascoid morphology has been studied, neuronal activity associated with this olfactory sensillum has not previously been recorded.

In the present investigation the selectivity of the responses of specific neurones within the ascoïd sensillum were employed to isolate and identify oviposition attractants in air-entrained volatiles of rabbit and chicken faeces.

Method and Materials

Sandflies. *L. longipalpis* employed in the investigation were originally collected from Jacobina, Bahia State, Brazil, and reared over approximately seventy-three generations at the Liverpool School of Tropical Medicine,

according to the method of Modi & Tesh (1983). Female flies used in the oviposition bioassay were blood-fed on hamsters anaesthetized with a 12 mg/ml solution of sodiumpentobarbitone. Sandflies were then isolated for 4 days with an equal number of males at 27°C, 95% r.h. and LD 12:12 h, to allow copulation, complete defecation and oogenesis. Sandflies used for electrophysiology experiments were 2–3 days old. All flies were allowed access to a solution of super-saturated sucrose, as provision of a sugar meal increases the number of eggs laid per sandfly (ElNaiem, 1991).

Biological extracts. Rabbit faeces were obtained from a pet rabbit fed on commercially available rabbit food, supplemented occasionally with green vegetables. Volatile components of the faeces were entrained onto a 4 mm i.d. glass capillary packed with 25 mg of Super Q adsorbent (80–100 mesh; Alltech, U.S.A.) between glass-wool plugs, using the method of Turlings *et al.* (1991). The volatile collection system employed air, first purified over an activated charcoal filter, which blew (1.5 l/min) the volatiles from 10 g of rabbit faeces in a closed Pyrex container (volume approx. 500 cm³) onto the adsorbent. After 2 h the collection trap was rinsed with 200 μl of dichloromethane. All solvents used in this investigation were pesticide analysis grade. The extract was concentrated for electrophysiology to 5 μl (standard concentration) by letting it stand at room temperature, and for bioassay to 5 μl on ice under a stream of nitrogen. A blank control

was prepared by carrying out an entrainment with an empty Pyrex vessel. *L. longipalpis* sex pheromone extracts were prepared by placing dissected tergal glands from twenty males in a tapered vial with 100 μ l of hexane, and the extract reduced in volume so that 1 μ l contained 1 male equivalent (Hamilton *et al.*, 1994).

Electrophysiology. The sandfly was anaesthetized with CO₂ and the legs removed with micro-scissors under a dissection microscope. The insect was then mounted on double-sided sticky tape on a microscope slide, with wings, thorax and abdomen pressed gently into place. It was necessary to use a black background (insulating tape) as ascoid sensilla were transparent with the incident cold-light source employed. Tungsten electrodes were mounted on Leitz micromanipulators, under a Leica M32 (Switzerland) combistereomicroscope. The reference electrode was inserted in the occipital sulculus of the sandfly, which provided good electrical contact and also kept the head stationary. The antenna was laid out on a second layer of double-sided sticky tape and carefully embedded in the glue, segment by segment, using a human eye lash held in an all-purpose scalpel blade holder. The tungsten recording electrode, with an electrolytically etched tip of 1 μ m, was gently introduced at an angle of 120° into the base of the ascoid until spontaneous neural activity was heard on the audio monitor of the main amplifier.

Recorded signals were passed through a preamplifier (10¹² Ω input impedance) into a universal AC/DC amplifier (UN-03, Syntech, The Netherlands) and recorded on one channel of a digital audio tape recorder (Biologic, DTR 1200, France). Signals were simultaneously displayed on an oscilloscope (Tektronix, LR 37158, U.S.A.). Hard copies of recorded data were obtained with the plot option of the spike analysis program for insect data, SAPID (Smith *et al.*, 1990), by playing back recordings from the DTR 1200 into a 386 IBM compatible personal computer via a DAS16 analogue/digital board (Metrabyte Corp., U.S.A., digitizing rate 10 kHz). Discrimination for the different cell types in the multicellular recordings from the ascoid sensillum was carried out by eye using spike amplitude, frequency and shape as criteria.

Electrophysiological stimulation. The electrophysiological preparation was maintained in a charcoal-scrubbed airflow at 1 m/s and 100% r.h. at 26 \pm 1°C by circulating the air through water-filled gas-wash bottles in a heated water bath. The exit of the glass tube (6 mm i.d.) conducting the air-flow was approximately 0.6 cm from the sandfly antenna. Stimulation was achieved with a charcoal-filtered air stream applied via a stopper on the barrel of a 5 ml polypropylene syringe containing the stimulus. A solenoid valve in the stimulus delivery system (ST-05, Syntec, Netherlands) permitted displacement of 2 ml of the syringe content in 1 s into the humidified air stream, through a septum-covered hole in the glass delivery tube at 20 cm from the outlet. For each stimulation the recorded pre-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 5 V TTL signal generated by the ST-05 when stimulation started was recorded on a second channel of the DTR-1200 and used as the 'point of stimulation' signal for subsequent analyses of responses.

The biological extracts and the following synthetic chemicals were at first employed to study the specificity of the receptors located in the ascoid sensillum: CO₂, H₂S, lactic acid, hexanol, hexylacetate, trans-2-hexen-1-al, cis-3-hexenylpyruvate, 1-octen-3-ol, trans-2-nonenal, octanoic acid, 10-undecenoic acid, γ -valerolactone, linalool, linalylacetate, geranyl acetate, citral, R(+)-limonene, α -terpinene, α -terpineol, terpinolene, (-)-menthol, carvacrol, trans-dihydrocarvone, R(+)- α -pinene; S(-)- α -pinene, R(+)- β -pinene, S(-)- β -pinene, α -copaene, bornylacetate, β -ionone, β -farnesene, β -elemene, β -carophyllene, γ -bisabolene, phenol, benzaldehyde, benzylacetate, methyl-salicylate, trans-methyl-iso-eugenol, elemicine, 1-methyl-naphthalene, 2-methyl-amino-methanol, hexylamine and 2-methoxy-butylpyrazine. Except for CO₂ (from a cylinder) and H₂S (from an aqueous Na₂S solution), all the chemicals (procured from commercial sources and >95% purity) were dissolved in dichloromethane and tested at 1 mg in the stimulus delivery syringe (unless otherwise stated). A 100 μ l aliquot of the solutions with synthetic products was applied to a 4 \times 1 cm filter paper strip. The extracts of biological origin were used at concentrations of 20 μ l of male gland extract (twenty male equivalents) and 1 μ l of faeces extract. After evaporation of the solvent, one drop of paraffin oil was applied to reduce evaporation of the test materials. Separate syringes were used for each stimulus and a delay of at least 30 min was allowed for evaporation within the syringe. Hexanal, 2-methyl-2-butanol and α (+)-pinene were used to stimulate the ascoid sensillum at doses of 10 μ g, 100 μ g and 1 mg, and hexanal plus 2-methyl-2-butanol were also tested combined in equal quantities of 1 mg in the odour delivery syringe. These three compounds were also used along with benzaldehyde in double successive stimulations. Stimuli were tested on at least one ascoid sensillum of a minimum of four flies.

Gas chromatography linked single sensillum recordings (GC-SSR). Olfactory receptors of the ascoid sensillum which had been shown to respond to some synthetic chemicals and to biological extracts were subsequently employed to locate any active products among the many constituents of the odours entrained from the faeces. Components of these biologically active extracts were separated by high-resolution capillary gas chromatography on a Carlo Erba chromatograph (HRGC 5160 Mega Series) equipped with an on-column injector and a flame ionization detector (FID). Samples of the rabbit faeces odour (2 μ l of standard concentrate) were injected on-column at 40°C, onto a 30 m DBwax fused silica capillary column (J. & W. Scientific, U.S.A., 0.25 mm i.d., 0.25 μ m film thickness) with the FID at 230°C. The column temperature was programmed from 40°C after 5 min at 8°C/min to 240°C. Hydrogen served as carrier gas at a flow rate of 1 ml/min. The column effluent was split 3:1 between the FID and the ascoid

sensillum via a heated transfer line (250°C) in the wall of the chromatogram. The conditioned air-stream (1 m/s), swept the effluent to the sensillum preparation 30 cm away. Column effluent was thus simultaneously monitored by the FID and olfactory receptors in the ascoid sensillum, in order to locate any active component(s) of the extract being analysed. Any change in the overall sensitivity of the preparation was monitored by applying $\alpha(+)$ -pinene as a standard stimulus.

All spikes from what usually amounted to multicellular recordings (AC signal) were sorted from background noise with 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 (time constant of the frequency to voltage converter: 1 s). This signal was printed on a multichannel chart-recorder simultaneous with the FID response. An electrophysiological response in GC-SSR was therefore indicated by a sudden change in the overall activity of the olfactory cells recorded.

Gas chromatography with mass selective detection (GC-MS). Extracts analysed by GC-SSR to locate active constituents were subsequently concentrated 100 times and analysed on the same GC phase (mentioned above) by GC-MS (Hewlett Packard 5890 series-II chromatograph—mass selective detector 5971A) to identify the active products. 2 μ l of extract were injected on-column to the DBwax capillary column, connected via 1 m of deactivated fused-silica capillary to the MS operating in the EI mode (ionization chamber temperature 180°C; ionization energy 70 eV). Helium was used as carrier gas under constant pressure (velocity 0.3 m/s at 40°C) and separation was achieved with the same temperature programme as in GC-SSR. Active components of the extracts located by GC-SSR were relocated in GC-MS with reference to simple alkanes injected under the same conditions, and by comparison of the chromatogram profiles obtained in GC-SSR and GC-MS. Identification of an electrophysiologically active peak in an extract was first based on the match of its mass spectrum with that of a known product stored in a computer-based library of the GC-MS, and subsequently on a match between the mass spectrum and retention time of the active peak and the synthetic analogue. Furthermore, using the single ion monitoring facility (SIM) of the mass selective detector, the presence of other olfactory stimulants in extracts was searched for at the retention time of the synthetic analogue using characteristic fragment ions of the products in question.

Gas chromatography. GC-FID analysis of the rabbit faeces odour extract was carried out on polar and non-polar phases for purposes of quantification, and to confirm that the retention time of the two products proposed as olfactory stimulants in the GC-MS analysis of extracts did indeed match that of synthetic analogues. The chromatograph (AI GC 93, AI Scientific, U.K.) was fitted with a Grob split/splitless injection system with a 30 s sampling time. The two fused-silica capillary columns used were both 30 m long, 0.25 μ m film thickness and 0.25 mm i.d., one of non-polar (DB1) and the other polar (DBwax, J. and W. Scientific). Both columns were temperature pro-

grammed from 35°C after 5 min at 8°C/min up to 80°C, held for 5 min and then at 10°C/min to 240°C. Carrier gas was helium at a flow rate of 1.4 ml/min and the injector and detector temperatures were 190°C and 220°C, respectively. Integration was carried out with the Varian Star Workstation and integrator board (A2 release, Varian Instruments, U.K.). 10 ng of hexanal and 2-methyl-2-butanol were injected onto each column and then 1 μ l of the rabbit faeces extract (standard concentration). The retention time of the synthetic standards and the two predominant early eluting peaks in the faecal odour extracts were compared on each column, over four injections.

Oviposition bioassay. The bioassay was an adaptation of the method used by Dougherty *et al.* (1993). Cored GC septa (Dyson Instruments, U.K.), heat sealed at one end, were placed into holes 10 cm apart in the plaster of paris base of a polymethylpentene larval rearing pot (11 cm diam \times 9.5 cm high, BDH, Nalgene). Both test and control septa were covered with a cotton-wool pad to prevent access by the flies, and to form a suitable oviposition substrate. A solution of the test chemical was placed dropwise into the test septum; the control septum had an equal volume of solvent alone placed into the core. The samples which were tested on separate days included entrained volatiles of rabbit and chicken faeces, hexanal, 2-methyl-2-butanol, hexanal/2-methyl-2-butanol, dimethyl disulphide, hexanal/dimethyl disulphide, $\alpha(+)$ -pinene and solvent controls. A 100-fold concentration (2 μ l) of rabbit and chicken faeces volatiles and 600 ng of pure compounds was used in each test. The combined mixture of hexanal and 2-methyl-2-butanol was also tested at 200 ng each and $\alpha(+)$ -pinene at 1 mg. Ten replicates were made of each test material and twenty gravid flies were used in each replicate. Eggs laid on the test and control pads were counted daily, for 4 days; eggs laid elsewhere on the plaster of paris base of the bioassay pot were not considered.

As the oviposition data were not normally distributed (Anderson-Darling normality test) the differences in the number of eggs laid at the test and control sites were statistically analysed using the Wilcoxon matched rank paired test. To test for differences between the treatments used in the bioassay, the number of eggs laid at the test site for each material was log transformed and subjected to a one-way ANOVA. Although tests with the different treatments were carried out with different lots of flies, normalization of the data before comparing treatments was deemed unnecessary due to the consistency of oviposition on controls between trials.

Results

Electrophysiological responses to biological extracts

The overall spontaneous activity within the sensillum was complex, with numerous cells firing. However, clear-cut responses were obtained to the two extracts of biological origin (Fig. 2). The response to the faecal odour was characterized by the inhibition of the cell producing the largest amplitude spikes. When stimulation stopped, this

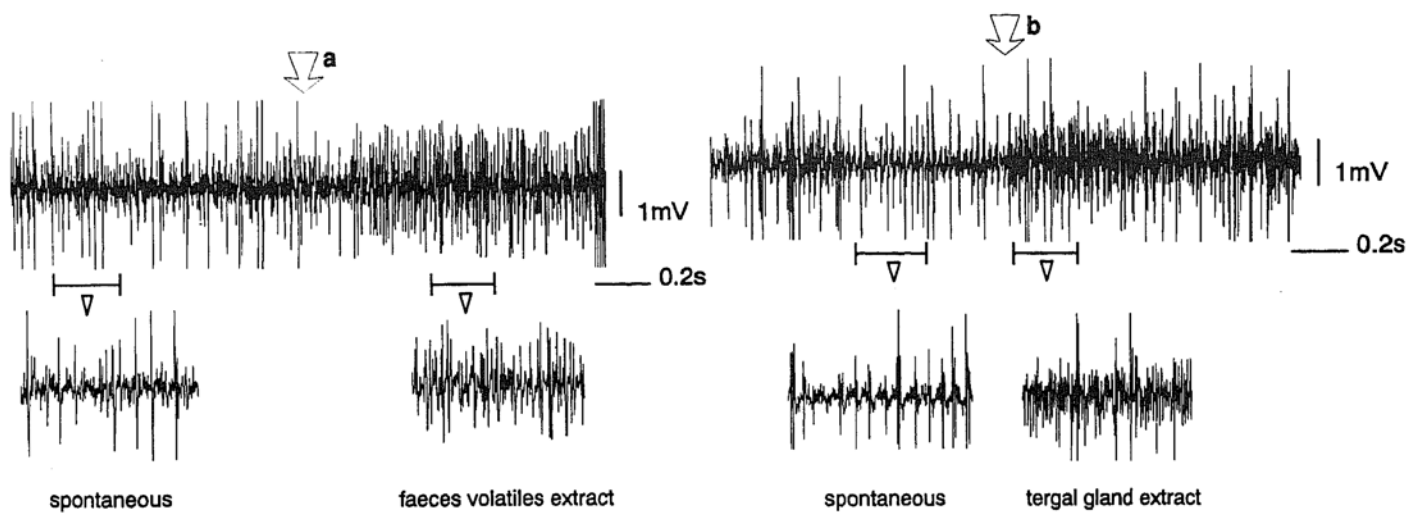


Fig. 2. Electrophysiological responses of olfactory cells within an ascoid sensillum on the antenna of a female *L. longipalpis* to faeces volatile extract (a) and to a male sandfly tergal gland pheromone extract (b). Open arrows on the upper traces in a and b show the start of stimulation which lasted 1 s; the lower traces are expanded sections of the upper traces.

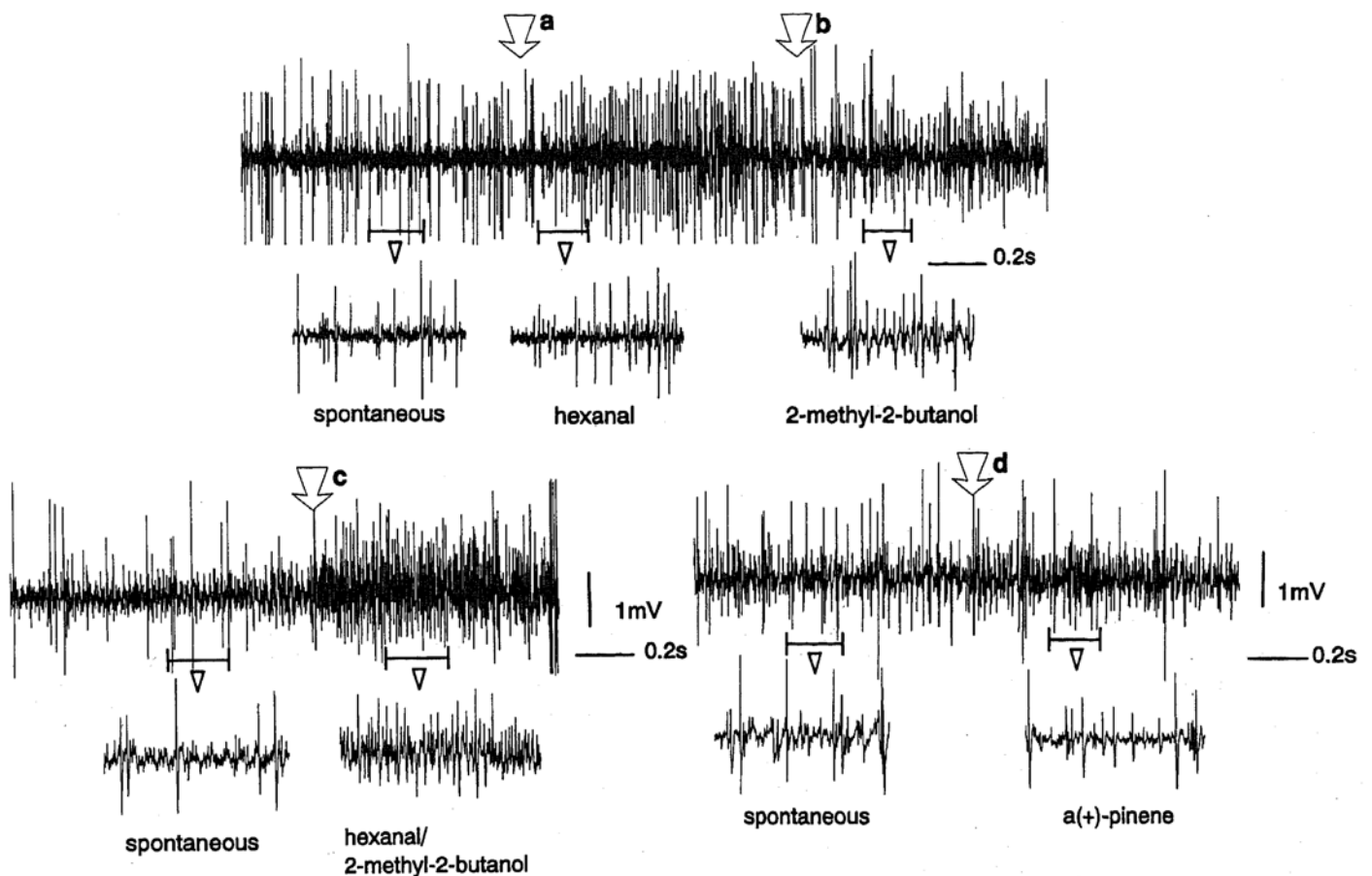


Fig. 3. Electrophysiological responses of olfactory cells within an ascoid sensillum on the antenna of a female *L. longipalpis* to double successive stimulation with hexanal (a) and 2-methyl-2-butanol (b), to a 1:1 mixture of hexanal plus 2-methyl-2-butanol (c) and to R(+)- α -pinene (d). Open arrows on the upper traces indicate the start of stimulation which lasted 1 s; the lower traces are expanded sections of the upper traces.

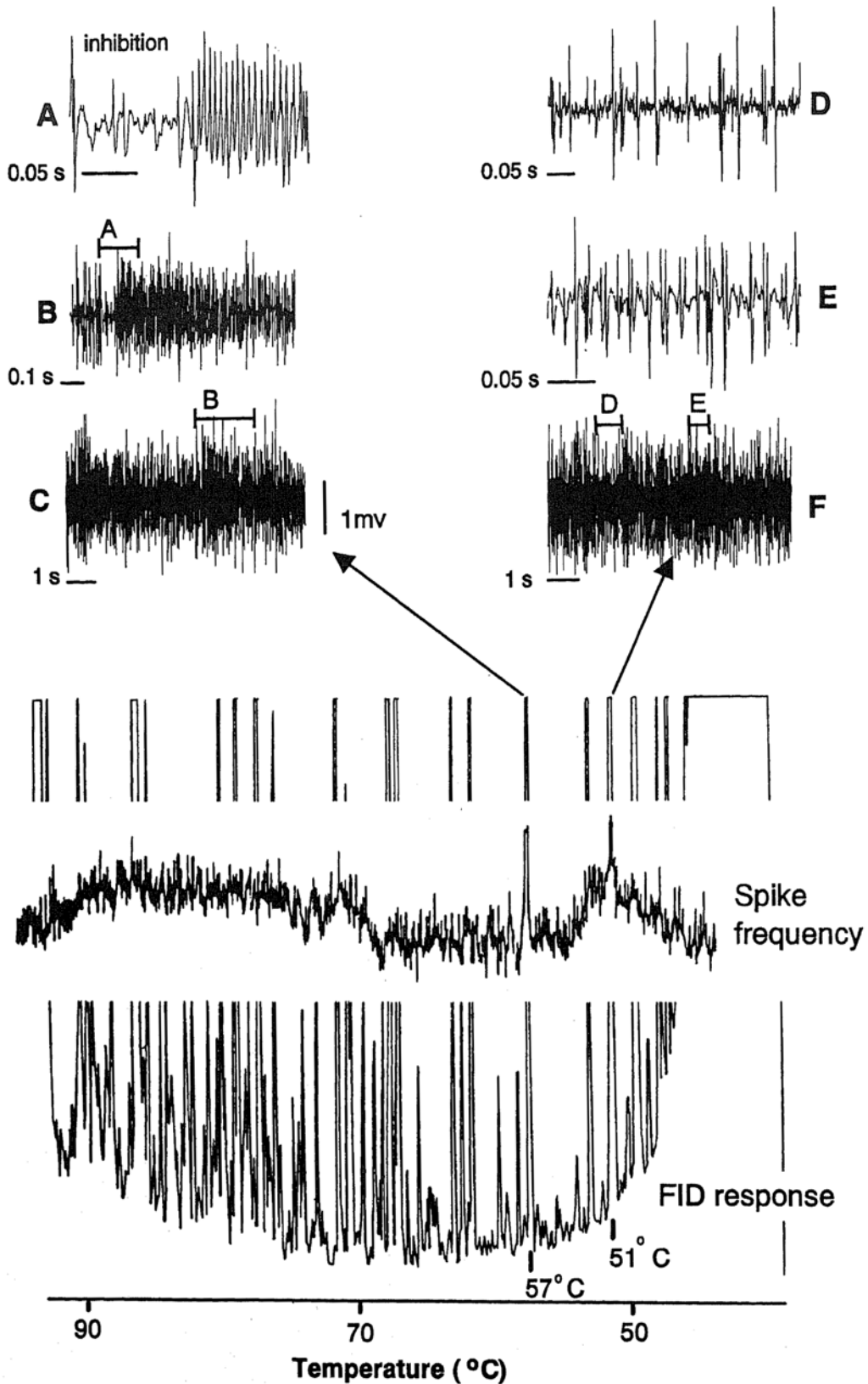


Fig. 4. Analysis of rabbit faeces volatiles by gas chromatography coupled electrophysiological recording from an ascoid sensillum of a female *L. longipalpis*. The bottom trace is the flame ionization detector response (FID). The spike frequency is the summed frequency of all firing cells (after frequency to voltage conversion of the spike train). The AC signal (upper traces) was also stored on a digital audio tape for subsequent analysis of responding cells during elution of the active peaks. The first active component of the extract eluted at 51°C (matching the retention time of 2-methyl-2-butanol) and caused an increase in cell activity (F), above the spontaneous level (D), of a cell characterized by a spike of intermediate amplitude (E). The second active peak eluted at 57°C (matching the retention time of hexanal). Elution of this peak caused a brief inhibition (B) and then a sustained burst of potentials (C). Detailed analysis of the spike discharge indicated that a second cell type was selectively activated (A) by this peak. (For gas chromatographic conditions, see text.)

cell responded with a rebound (Fig. 2a). In contrast, two other cells producing spikes of intermediate amplitudes increased their firing rates. The tergal gland extract, containing the sex pheromone, appeared only to affect the activity of one cell type which was characterized by a small amplitude spike showing a predominantly negative going component (Fig. 2b).

Electrophysiological responses to synthetic volatiles

Eight compounds elicited a response from olfactory receptors in the ascoïd sensillum: R(+)- α -pinene, S(-)- α -pinene, R(+)- β -pinene, S(-)- β -pinene, α -terpinene, benzaldehyde, hexanal and 2-methyl-2-butanol (Fig. 3). Hexanal and benzaldehyde both activated a cell with spikes characterized by a relatively big amplitude and roughly equal positive and negative going components (Fig. 3a). Both products also mimicked the faeces odour extract by inhibiting the cell with the largest spike amplitude. Hexanal was the most active synthetic tested, as it was the only product to activate a receptor at 100 μ g source under paraffin oil. This receptor had a higher threshold for benzaldehyde. Double successive stimulation showed that the response to 2-methyl-2-butanol was very different to that of hexanal (Fig. 3b), with lower amplitude spikes predominating. This suggests the presence of separate receptors for hexanal and 2-methyl-2-butanol within the ascoïd sensillum. Stimulation with R(+)- α -pinene increased the firing frequency of what appeared to be a fourth receptor characterized by a small amplitude spike with a predominantly positive-going component (Fig. 3d). The stimulation threshold was rather high, because no response was observed to the pinene isomers at less than a 1 mg source. All the pinene isomers tested appeared to be equally active. α -Terpinene also induced an increase in the firing rate of the cell responding to the pinene isomers. The cell with the small negative-going spike which responded to the tergal gland extract did not respond to any of the synthetic chemicals tested. Stimulation with air or solvents caused no significant increase in spike frequency of any of the ascoïd cells.

Gas chromatography-linked single sensillum recordings

Two active constituents of the rabbit faeces volatile extract were located by GC-SSR (Fig. 4). The first active peak eluted at 51°C and matched the retention time of 2-methyl-2-butanol on the two GC phases employed (see below). This peak activated the receptor type which responded to synthetic 2-methyl-2-butanol. During elution of the second active component, at 57°C, a brief inhibition was followed by an increase in frequency of the spike which characterized the response to hexanal. The peak at 57°C matched the retention time of hexanal on the two GC phases employed (see below).

GC-MS analysis

Analysis of both rabbit and chicken faeces extracts by GC-MS confirmed the presence of 2-methyl-2-butanol (M^+ 88) and hexanal (M^+ 100) as major components in the early part of the chromatogram. The mass spectra and retention times of the electrophysiologically active constituents of the faecal extracts matched those of synthetic 2-methyl-2-butanol and hexanal. Single ion monitoring indicated the presence of benzaldehyde at levels approximately half that of hexanal in both the rabbit and chicken faeces volatile extracts. Dimethyl disulphide was detected in volatiles of chicken faeces but not in rabbit faeces. None of the pinene isomers or α -terpinene were detected in the faeces extracts.

GC-FID analysis

On the nonpolar phase 2-methyl-2-butanol eluted at 4.287 min matching the first active peak at 4.28 min, and hexanal eluted at 10.02 min matching the second active component which eluted at 10.02 min. The match between the active peaks in the extract and the two synthetic compounds was equally high on the polar phase: active peak 1 eluted at 9.632 min and 2-methyl-2-butanol at 9.631 min, whereas active peak 2 eluted at 11.763 min and hexanal at 11.770 min. The two active peaks constituted major components in the early part of the chromatogram on both phases, with 2-methyl-2-butanol at 60 ng/ μ l and hexanal at 40 ng/ μ l of concentrated extract.

Oviposition responses

The extract of volatiles from rabbit and chicken faeces produced an oviposition response from gravid flies, in that significantly more eggs were laid at the test site than the control (Table 1). No significant difference was observed between egg counts for rabbit and chicken faeces volatiles. The blank control, i.e. entrainment from an empty Pyrex vessel, had no effect on oviposition choice. Hexanal evoked an oviposition response that yielded significantly more eggs at the test site than the control, whereas 2-methyl-2-butanol did not. Hexanal alone did not produce an oviposition response as high as either the rabbit or chicken faeces volatile extracts, but when hexanal was applied in conjunction with the 2-methyl-2-butanol, oviposition at the test septum was statistically comparable to the faecal volatile extracts. A 1:1 mixture of 2-methyl-2-butanol and hexanal at 400 ng, 3 times lower than the dose of the mixture listed in Table 1, stimulated oviposition at the test septum (197 ± 14.2 test: 40.7 ± 8.2 control, $P = 0.001$) to the same extent as an aliquot of faeces volatiles containing roughly comparable amounts of these two compounds. Dimethyl disulphide neither attracted gravid females for oviposition on its own nor did it alter the efficacy of hexanal when tested together. Pinene attracted gravid flies

Table 1. Oviposition response of *L. longipalpis* to test septa treated with entrained volatiles of rabbit and chicken faeces, hexanal, 2-methyl-2-butanol, dimethyl disulphide, and 1:1 mixtures of hexanal plus 2-methyl-2-butanol and hexanal plus dimethyl disulphide (each synthetic substance at a dose of 600 ng). Numbers of eggs laid at the test and control (solvent alone) septa were compared using the Wilcoxon matched rank paired test ($n = 40$). A comparison between treatments (vertical) was carried out, after a logarithmic transformation of the data, by ANOVA; treatments with the same letter are not significantly different.

Material bioassayed	Mean number of eggs laid \pm SE		P	Comparison of treatments
	Test	Control		
Rabbit faeces volatiles	224.3 \pm 13.8	42.7 \pm 11.7	0.006	a
Chicken faeces volatiles	185.5 \pm 15.5	37.7 \pm 7.9	0.007	a
Solvent control	31.8 \pm 14.5	35.2 \pm 10.8	0.410	c
Hexanal	126.4 \pm 13.5	39.4 \pm 9.6	0.006	b
2-Methyl-2-butanol	27.5 \pm 3.25	34.4 \pm 7.3	0.221	c
Dimethyl disulphide	34.6 \pm 8.62	42.3 \pm 12.2	0.262	c
Hexanal/2-methyl 2-butanol	212.4 \pm 13.6	38.6 \pm 10.6	0.006	a
Hexanal/dimethyl disulphide	127.3 \pm 15.2	41.4 \pm 9.2	0.011	b

for oviposition only when present on the septa at 1 mg (98.3 \pm 9.7 test: 33.6 \pm 8.81 control, $P = 0.041$).

Discussion

This is the first electrophysiological investigation on olfactory receptors of phlebotomine sandflies and confirms the ascoid sensillum on the antennae of *L. longipalpis* as a significant olfactory organ. Receptor cells in this sensillum respond to behaviourally active odour extracts of faeces from rabbits and chickens, and to a sex pheromone extract. Two constituents of these faecal extracts, i.e. 2-methyl-2-butanol and hexanal were identified as olfactory stimulants by GC-SSR. In oviposition bioassays these two synthetic volatiles combined were as active as an aliquot of faeces odour containing similar amounts of these products. R(+)- α -pinene, another olfactory stimulant, did attract gravid flies in the oviposition bioassay, but only when present at relatively high amounts. This compound was not detected in the faecal extracts. Furthermore, in contrast to the response with either hexanal or the hexanal/2-methyl-2-butanol mixture, the oviposition response from the gravid flies was not as targeted on the cotton-wool pad, i.e. many eggs were also laid on the plaster of paris around the test septum when pinene was applied, even though present at a quantity of 1 mg.

The response of ascoid sensilla to extracts of faeces volatiles was typified by inhibition of the receptor with the largest spike amplitude and stimulation of at least two other receptor cells. Hexanal, the product for which the lowest threshold was recorded, also inhibited the cell with the largest spike amplitude but activated another. 2-Methyl-2-butanol did not cause inhibition but activated a third cell type. Despite the complexity of the multicellular response recorded from the sensillum, double successive stimulation also strongly suggested that separate cells were being activated by hexanal and 2-methyl-2-butanol.

To date, only extracts of whole rabbit faeces solids have been shown to induce oviposition attraction in gravid female flies (ElNaiem & Ward, 1992b; Dougherty *et al.*, 1993). Now that some active components of faecal volatiles have been identified, further work is clearly needed to establish if these compounds can enhance the oviposition attraction of egg-associated products isolated by Dougherty *et al.* (1994). An extract of rabbit faeces solids combined with the oviposition pheromone act synergistically on *L. longipalpis* oviposition, with enhanced egg laying and post oviposition survival. Furthermore, in nature, sandflies have been observed to emerge in great numbers into traps placed over cracks and fissures in livestock pens, places of relatively stable humidity and an abundant supply of faeces (Bettini, 1988). The oviposition pheromone erythro-6-acetoxy-5-hexadecanolide and stagnant water have a similar additive effect on the oviposition behaviour of *Culex quinquefasciatus* Say, with egg laying greatly stimulated when exposed to the combined semiochemical sources (Mordue *et al.*, 1992).

A number of other volatiles were found to activate cells within the ascoid sensillum, namely R(+)- α -pinene and some of its isomers, α -terpinene and benzaldehyde. The pinenes were the first synthetic stimuli we found for olfactory receptors of *L. longipalpis*, hence their use as a standard in GC-SSR. There were only small differences in the degree of stimulation induced by the pinene isomers, and all stimulated the same receptor type. The same cell responded to α -terpinene. None of these compounds was detected by GC-MS in the faeces volatile extracts. However, it is possible that these simple terpenes play a role in the orientation of sandflies to plants as these insects are known to take sugar meals in the wild and even exhibit plant preferences (Cameron & Davies, 1993). Mosquitoes also use plant nectar as an energy source, and terpene-sensitive olfactory receptors have been identified on the antennae of *Culex pipiens* (Bowen, 1992).

Benzaldehyde activated the same olfactory cell as

hexanal, and likewise inhibited the cell with the largest spike amplitude. Selective ion monitoring indicated the presence of this aromatic product at levels approximately half that of hexanal in both the rabbit and chicken faeces volatile extracts. The lower level of the compound in the extracts, combined with the overall higher threshold of the receptor for benzaldehyde than for hexanal, may go some way to explain why no response was recorded for this product in GC-SSR. Since hexanal and benzaldehyde both possess a carbonyl group it is not surprising that they activate the same cell. However, other aliphatic aldehydes failed to activate the receptor in the electrophysiology experiments. This would tend to suggest that perception of benzaldehyde is of some significance for the sandfly. Benzaldehyde has been identified as a host-odour olfactory stimulant for a tick parasite of bovids (Steullet & Guerin, 1994), and this product is a well-known aromatic constituent of flower blossoms (Knudsen *et al.*, 1993). Further work is needed to establish the behaviour stimulated by benzaldehyde, if any, on its own and in combination with the other stimulants identified.

Although α -terpineol did not stimulate any cells of the ascoid sensillum, it has been shown to be an attractant for another species of sandfly, *Phlebotomus papatasi* (Wilson *et al.*, 1989a). Other sensilla which may play a role in olfaction are present on the antenna of the sandfly and could serve to perceive semiochemicals, or, alternatively, this compound may be important to *P. papatasi* but not to *L. longipalpis*. Other compounds identified as attractants for *Phlebotomus* species are dibutyl succinate, dimethyl disulphide and mixtures of these two compounds (Wilson *et al.*, 1989b, 1990). Dimethyl disulphide was detected in the extract of chicken faeces volatiles, but failed to attract gravid *L. longipalpis* for oviposition, or enhance the activity of hexanal.

One receptor characterized by a small amplitude spike and a predominantly negative-going component was stimulated by the tergal gland extract of *L. longipalpis* males. This species is a homosesquiterpene type phlebotomine from Jacobina, Brazil producing a one component sex pheromone (Hamilton *et al.*, 1994). This is supported by the electrophysiological responses reported here where only one olfactory cell was stimulated. Identification of the neurone that responds to the sex pheromone should enable an electrophysiological investigation of the *L. longipalpis* species complex. Problems which could be investigated by electrophysiology include male perception of the sex pheromone, pheromone sensitivity across species, and the screening of synthetic pheromone analogues.

It is now possible to record electrophysiological activity from olfactory neurones of these small insects, providing another tool for investigating their use of semiochemicals. A laboratory oviposition trap has been developed using extracts of whole faeces and *L. longipalpis* oviposition pheromone, and the trap has been used to collect either gravid females or eggs (Dougherty *et al.*, 1993). In conclusion, as much is unknown about the behaviour of these important disease vectors in the field, the identification of these volatiles as oviposition attractants will improve the

oviposition trap. This may allow its use to gather basic information on the biology and ecology of the sandfly and monitor populations.

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References

- Ashford, R.W., Desjeux, P. & DeRaadt, P. (1992) Estimation of population at risk of infection and number of cases of leishmaniasis. *Parasitology Today*, **8**, 104–105.
- Bettini, S.C. (1988) Sandfly breeding sites. *Leishmaniasis, the Current Status and New Strategies for Control* (ed. by D. T. Hart), pp. 179–188. Plenum Press, New York.
- Bettini, S.C. & Melis, P. (1988) Leishmaniasis in Sardinia. III. Soil analysis of a breeding site of three species of sandflies. *Medical and Veterinary Entomology*, **2**, 67–71.
- Boufana, B.S. (1990) The tergal pheromone gland and antennal sensilla of the sandfly *Lutzomyia longipalpis*. Ph.D. thesis, University of Liverpool.
- Bowen, M.F. (1992) Terpene-sensitive receptors in female *Culex pipiens* mosquitoes: electrophysiology and behaviour. *Journal of Insect Physiology*, **38**, 759–764.
- Cameron, M.M. & Davies, C.R. (1993) Ecological studies support a key role for aphid honeydew as a nutrient source for sandflies. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **87**, 363.
- Dougherty, M.J., Hamilton, J.G.C. & Ward, R.D. (1993) Semiochemical mediation of oviposition by the phlebotomine sandfly *Lutzomyia longipalpis*. *Medical and Veterinary Entomology*, **7**, 219–224.
- Dougherty, M.J., Hamilton, J.G.C. & Ward, R.D. (1994) Isolation of oviposition pheromone from the eggs of the sandfly *Lutzomyia longipalpis*. *Medical and Veterinary Entomology*, **8**, 119–124.
- Dougherty, M.J., Ward, R.D. & Hamilton, G. (1992) Evidence for the accessory glands as the site of production of the oviposition attractant and/or stimulant of *Lutzomyia longipalpis* (Diptera: Psychodidae). *Journal of Chemical Ecology*, **7**, 1165–1175.
- Dye, C., Guy, M.W., Elkins, D.B., Wilkes, T.J. & Killick-

- Kendrick, R. (1987) The life expectancy of phlebotomine sandflies: first field estimate from southern France. *Medical and Veterinary Entomology*, **1**, 417–425.
- ElNaiem, D.A. (1991) Oviposition of *Lutzomyia longipalpis* (Diptera: Psychodidae) and development of *Leishmania chagasi* (Kinetoplastida: Trypanosomatidae) in the vector. Ph.D. thesis, University of Liverpool.
- ElNaiem, D.A. & Ward, R.D. (1992a) The thigmotropic oviposition response of the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae) to crevices. *Annals of Tropical Medicine and Parasitology*, **86**, 425–430.
- ElNaiem, D.A. & Ward, R.D. (1992b) Oviposition attractants and stimulants for the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae). *Journal of Medical Entomology*, **29**, 5–12.
- Hamilton, J.G.C., Dougherty, M.J. & Ward, R.D. (1994) Sex pheromone activity in a single component of tergal gland extract of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Jacobina, Northeastern Brazil. *Journal of Chemical Ecology*, **20**, 141–151.
- Knudsen, J.T., Tollsten, L. & Bergström, L. (1993) Floral scents: a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry*, **33**, 253–280.
- Modi, G.B. & Tesh, R. (1983) A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. *Journal of Medical Entomology*, **5**, 568–569.
- Mordue, A.J., Blackwell, B.S., Hasson, B.S., Wadhams, L.J. & Pickett, J.A. (1992) Behavioural and electrophysiological evaluation of oviposition attractants for *Culex quinquefasciatus* Say (Diptera: Culicidae). *Experientia*, **48**, 1109–1111.
- Smith, J.J.B., Mitchell, B.K., Rolseth, B.M., Whitehead, A.T. & Alben, P.J. (1990) SAPID tools: microcomputer programs for analysis of multi-unit nerve recordings. *Chemical Senses*, **15**, 253–270.
- Steullet, P. & Guerin, P.M. (1994) Identification of vertebrate volatiles stimulating olfactory receptors on tarsus I of the tick *Amblyomma variegatum* Fabricius (Ixodidae). I. Receptors within the Haller's organ capsule. *Journal of Comparative Physiology*, **A**, **173**, 27–38.
- Turlings, T.C.J., Tumlinson, J.H., Heath, R.R., Provcaux, A.T. & Doolittle, R.E. (1991) Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. *Journal of Chemical Ecology*, **17**, 2235–2251.
- Ward, R.D. (1974) Studies on the adult and immature stages of some phlebotomid sandflies (Diptera: Psychodidae) in Northern Brazil. Ph.D. thesis, University of London.
- Wilson, R.A., Butler, J.F., Withycombe, D., Mookherjee, B.D., Katz, I. & Schrankel, K.R. (1989a) Use of dibutyl succinate, dimethyl disulphide and mixtures of same as insect attractants. United States Patent 4801448, granted 31 January 1989.
- Wilson, R.A., Butler, J.F., Withycombe, D.A., Mookherjee, B.D., Katz, I. & Schrankel, K.R. (1989b) Use of alpha-terpineol as insect attractant. United States Patent, 4886662, granted 12 December 1989.
- Wilson, R.A., Butler, J.F., Withycombe, D.A., Mookherjee, B.D., Katz, I. & Schrankel, K.R. (1990) Use of dibutyl succinate as an insect attractant. United States Patent 4911906, granted 27 March 1990.