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Sensory and behavioural responses of the stable fly *Stomoxys calcitrans* to rumen volatiles

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Abstract. Analysis of volatiles from rumen digesta by gas chromatography linked antennogram recordings from *Stomoxys calcitrans* (L) (Diptera: Muscidae) antennal receptor cells revealed about 30 electrophysiologically active constituents, the most important of which is dimethyl trisulphide with a sensory threshold in the femtogram range. The behavioural responses of *S. calcitrans* to five chemostimulants (dimethyl trisulphide, butanoic acid, *p*-cresol, oct-1-en-3-ol and skatole) were tested in a wind tunnel where activation and attraction of hungry flies to rumen volatiles were recorded. Dimethyl trisulphide, butanoic acid and *p*-cresol were found to attract *S. calcitrans*. This sensitivity to rumen volatile constituents, that also occur in animal wastes used for oviposition by *Stomoxys* spp., as well as in flowers used by stable flies as sources of nectar is discussed in the context of the behavioural ecology of these flies.

Key words. *Stomoxys calcitrans*, electroantennogram, olfaction, rumen digest, stable fly, wind tunnel.

Introduction

Although *Stomoxys calcitrans* will feed quite readily on a wide variety of hosts such as suids, canids, felids and even humans (Hafez & Gamal-Eddin, 1959; Zumpt, 1973), as a serious pest the stable fly is mainly considered to affect cattle and horses. Livestock facilities with many animals in confined areas provide readily accessible sources of food for blood-seeking flies, as well as manure for ovipositing females.

Haematophagous arthropods depend on blood for reproduction and rely on chemical and physical stimuli to find hosts (Cork, 1996; Gibson & Torr, 1999). Many of the chemostimuli implicated in host location originate from bacterial activity and are released from specific regions of the host body (Braks *et al.*, 1999). Human skin emanations are composed of numerous volatiles that affect mosquito behaviour (Bernier *et al.*, 2000; Meijerink *et al.*, 2000). Vale (1980) reported the attractiveness of ox body odours to some bloodsucking flies. Another important source of volatiles is breath; human breath has been shown to activate stable flies (Warnes & Finlayson, 1985) and mosquitoes (Healy & Copland, 1995), and attract ticks (McMahon & Guerin, 2002). Although the attractiveness of breath to bloodsucking insects is often associated with its carbon dioxide (CO_2) content, breath also contains numerous volatile compounds (Wahl *et al.*, 1996; Phillips *et al.*, 1999), and rumen volatiles expelled by eructation in bovine breath attract ticks (Donzé *et al.*, 2004).

Here we describe the sensitivity of *S. calcitrans* antennal receptor cells for rumen volatiles by recording antennogram responses to these products eluted from a gas chromatographic column. Responses of *S. calcitrans* to five important chemostimuli in rumen odour were tested in a wind tunnel. Their role in resource location by the stable fly is discussed.

Materials and methods

Insects and oviposition substrates

Stomoxys calcitrans pupae were obtained from Novartis Animal Health S.A. (St-Aubin, Switzerland). Newly emerged flies were sexed daily and approximately 30 flies were enclosed in cotton mesh cages (15 × 25 × 15 cm). Flies had access to cotton wetted with 10% sucrose solution in a plastic dish, changed daily. Experiments were conducted with 1–2-day-old unmated sugar-fed flies.

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Rumen volatile collection

Fresh rumen contents were collected, as needed, from mainly grass-fed steers at a slaughterhouse (La Chaux-de-Fonds, Switzerland) and 50 g aliquots were introduced into a 1-L gas-wash flask, flushed with nitrogen gas (N_2) in order to avoid oxidization of the substrate, capped with a glass T-connector and left for 1 h to equilibrate at room temperature. N_2 was blown into the bottle at 50 mL/min for 20 min and the headspace volatiles were collected at the outlet of the T-connector on a commercial Tenax™ GR cartridge with a 6-mm outer diameter (Gerstel® GmbH & Co., Muelheim an der Ruhr, Switzerland) selected for its relatively low affinity for water.

Electroantennograms

Electroantennograms (EAGs) from antennae of sugarfed *S. calcitrans* were recorded as in Guerin & Visser (1980). Each antenna was maintained in a humidified charcoal-filtered air stream (90–100% RH, 23 ± 2° C) delivered at 1 m/s via a glass water-jacketed tube (7 mm internal diameter) whose outlet was about 1 cm from the preparation. The EAG signal was recorded on a computer via a high impedance pre-amplifier (Syntech, Hilversum, the Netherlands), a DC amplifier (UN-03; Syntech) and an analogue-digital converter (USB-IDAC box; Syntech). Antennal stimulation was carried out as described in Guerenstein & Guerin (2001) by passing 1 mL of charcoal-filtered air in 1 s through a 5-mL polypropylene syringe (BD Plastipak™, Madrid, Spain) containing the stimulus into the air stream (above). Serial dilutions in methylene chloride (DCM, analytical grade; Merck, Glattbrugg, Switzerland) were prepared for each chemical tested and a 10-μL aliquot was deposited on a small filter paper strip (0.8 × 3 cm) that was inserted into the stimulus syringe after solvent evaporation. Given the amount evacuated from the stimulus syringe, the dilution in the humidified air stream and the surface area of the antenna, the relative amount of a synthetic product reaching the antenna was estimated to correspond to approximately 10^{-4} of the amount placed on the filter paper.

Gas chromatography linked electroantennographic detection

Electroantennogram detector (EAD) responses from sugar-fed stable flies to constituents of rumen volatile extracts were recorded in parallel with the flame ionization detector of the gas chromatograph (GC 5300; Carlo Erba Instruments, Milan, Italy) (for further details cf. Jeanbourquin & Guerin, 2007). Two types of column were used for the analyses of rumen volatiles by gas chromatography (GC) linked EAD: a 30-m long polar Free Fatty Acids Phase (FFAP) with an internal diameter of 0.25 mm and film thickness of 0.25 μm, and a 30-m long apolar SE-30, with an internal diameter of 0.25 mm and film thickness of 0.15 μm (both supplied by BGB Analytik, Boeckten, Switzerland). Rumen volatiles trapped on a Tenax™ GR cartridge were thermally desorbed as described elsewhere (Jeanbourquin & Guerin, 2007). The temperature programme stipulated 40° C for 5 min, rising at 5° C/min to 230° C for the FFAP phase, and 40° C for 5 min, rising at 3° C/min to 120° C, 5° C/min to 200° C and 8° C/min to 280° C for the SE-30 phase. An averaged GC-EAD trace was redrawn from the three most representative GC-EAD analyses of the rumen odour extract on the SE-30 column. Only antennal responses to a given constituent of the extract recorded in at least two GC-EAD analyses were considered. The same fly antenna was used to record the EAD responses to similar amounts of six rumen volatiles injected at successively increasing doses (0.2, 20 and 100 ng) on to the FFAP phase (conditions as above). The relative amount of a product reaching the antenna has been estimated to correspond to approximately $16*10^{-3}$ of the amount injected on-column, taking into account the effluent split ratio, the dilution in the humidified air stream and the surface area of the antenna.

Gas chromatography linked mass spectrometry

Biologically active constituents of rumen digesta that induced EAG responses from *S. calcitrans* antennae were identified by GC linked mass spectrometry (GC-MS) as described (Jeanbourquin & Guerin, 2007).

Wind tunnel experiments

The wind tunnel set-up described by Jeanbourquin & Guerin (2007) was used with the following modifications. The nylon flight cylinder connecting the odour source to the fly release point was removed so that flies could undertake free-flying behaviours. As stable flies are known to fly preferentially near the ground, the delivery funnels were lowered to 8 cm above the floor of the tunnel, as was the insect release point. A bigger cylindrical release cage made of 3-mm metal mesh (10 cm in diameter, 15 cm long) lying on its side was fitted with a 1.3-mm metal mesh flap attached to the cage on a hinge to allow the release cage to be opened using a string on a pulley from outside the wind tunnel. To permit orientation of flies in the wind tunnel, a pale blue cardboard strip (6 cm wide, 0.2 mm high) was attached in the centre of the wind tunnel floor, extending from the odour release point to the fly release cage. Two narrower strips (1 cm high) were also attached to the side walls of the wind tunnel 6 cm above the floor. The fly release cage with five unfed flies was placed in the wind tunnel and flies were allowed a 3-min acclimatization period in odour-free air. The release cage flap was then gently lifted and flies were exposed for 3 min to the test or control stimuli. The flight behaviour of each fly was recorded using OBSERVER software (Noldus Technologies, Wageningen, the Netherlands). Categories included: activation (i.e. leaving the release cage) and attraction (i.e. flying more than 50 cm upwind of the 130 cm distance between the release point and the odour source). Fly responses to test and control odours (see below) were examined separately and compared using chi-square tests on the actual counts. Results are presented as the percentages of flies activated and attracted for clarity. In addition, the mean number of orientated

flights (any sinuous flight in the odour plume of at least 50 cm in the direction of the odour source) per activated fly was calculated from the total number of orientated flights exhibited by any fly during 3 min. Differences in the number of these flights shown by flies between treatments were compared using the Mann–Whitney test. As no significant differences in either activation, attraction or the number of orientated flights made by male and female *S. calcitrans* to the different treatments were recorded ($P > 0.5$), data for the two sexes were pooled.

Test stimuli

A 50 g sample of rumen contents (test) from a freshly slaughtered steer or 50 mL distilled water (control) were introduced into 1-L gas-wash flasks and capped with glass T-connectors. One end of the T-connector was linked to a stimulus delivery system (Syntech) blowing charcoal-filtered air at 450 mL/min through the bottle via Teflon connections. The other end of the T-connector (exhaust) was linked to the aluminium tube with the funnel through which the odour entered the wind tunnel (Jeanbourquin & Guerin, 2007). The CO_2 concentration was measured at the funnel exit and at the fly release point with a Li-820 gas analyser (Li-Cor®, ± 5 p.p.m. resolution, LI-COR Biosciences, Lincoln, NE, U.S.A.).

Oct-1-en-3-ol (> 97%; Merck), dimethyl trisulphide (> 98%; Sigma-Aldrich, Buchs, Switzerland), *p*-cresol (> 99% puriss. p.a.; Fluka, Buchs, Switzerland), butanoic acid (> 99.5%; Fluka) and skatole (> 99%; Fluka) were diluted in dichloromethane (DCM) to obtain solutions of 100 ng/μL. Polyethylene dispensers (1.3 cm in diameter, 3.0 cm in length, with 0.8-mm thick walls; Kartell, Milan, Italy) were filled with 100 μL of these solutions, corresponding to 10 μg of each test substance, and placed open in a 1-L gas-wash bottle through which charcoal-filtered air passed at 400 mL/min. A dispenser containing 100 μL DCM was used as control.

Results

GC-EAD analysis of rumen odour

Rumen digesta was shown to contain many volatile compounds that elicit responses from *S. calcitrans* antennal receptor cells in GC-EAD analyses (Tables 1 and 2). Identification of 23 of the 36 compounds evoking EAD responses was made by GC-MS and confirmed by tests showing electrophysiological activity of the synthetic analogues. The identities of the remaining 13 compounds remain unconfirmed (Tables 1 and 2). Electroantennogram detector responses to co-eluting products were not attributed to any one product but to the compounds determined under the peak by GC-MS (i.e. peaks 4 and 8 on the SE-30 phase; Table 1) and peaks 9, 12 and 14 on the FFAP phase; Table 2) (Fig. 1). Analysis of the extract of rumen volatiles on the polar column revealed carboxylic acids, many aliphatic alcohols and several ketones as the dominant chemostimuli (Table 2). Among the other identified rumen constituents eliciting EAD responses were terpenes (citronellene, D-limonene, β-cyclocitral, geranyl acetone, α-humulene and β-caryophyllene), aromatics (*p*-cresol and skatole), decanal and dimethyl trisulphide. The highest EAD amplitudes were recorded to oct-1-en-3-ol, dimethyl trisulphide and β-cyclocitral, despite being present at between 1 ng and 10 ng in the extract aliquot, suggesting *S. calcitrans* olfactory receptor cells, have a low sensory threshold for these products.

Table 1. Compounds, identified by gas chromatography (GC) linked mass spectrometry, recovered from rumen digesta headspace that evoked responses from *Stomoxys calcitrans* antennae during GC electroantennographic detector (EAD) analysis (Fig. 1) on the apolar column (see Materials and methods).

Compound name	% ± SE	RI(1)	RI(2)	EAD response *n*°	Mean EAD ± SE (mV)
2-ethylhexan-1-ol	4.1 ± 0.6	1018	1015	4	1.24 ± 0.70
Decanal*	0.4 ± 0.1	1184	1186	8	2.25 ± 0.30
Acetophenone*	0.4 ± 0.1	1034	1100	5	0.82 ± 0.33
Skatole*	7.5 ± 0.3	1341	1370	9	0.65 ± 0.22
p-cresol*	57.0 ± 1.8	1042	1213	6	0.39 ± 0.07
Dimethyl trisulphide*	0.7 ± 0.4	942	949	1	2.28 ± 0.69
Citronellene*	8.2 ± 0.1	951	–	2	3.02 ± 0.38
D-limonene*	4.1 ± 0.6	1018	1022	4	1.24 ± 0.70
Dihydrocarvone	0.3 ± 0.0	1171	–	7	1.52 ± 0.22
β-cyclocitral*	0.4 ± 0.1	1184	1233	8	2.25 ± 0.30
β-caryophyllene*	9.0 ± 0.6	1396	1438	10	1.13 ± 0.26
Geranyl acetone*	0.2 ± 0.0	1424	1431	12	1.34 ± 0.62
α-humulene*	1.9 ± 0.3	1433	1472	13	1.04 ± 0.29
3,7-dimethyl-(*Z*)-oct-2-ene	9.9 ± 0.2	972	–	3	2.86 ± 0.70
Unidentified	0.2 ± 0.0	1414		11	1.27 ± 0.18

*Synthetic analogue showed electrophysiological activity in GC-EAD analyses or EAG recordings from *S. calcitrans.*
SE, standard error: represents the SE calculated from three GC-EAD analyses on the apolar GC phase.
RI(1), retention index of natural product.
RI(2) retention index of injected synthetic analogue in gas chromatography linked mass spectrometry analyses or RI from the literature (www.flavornet.org).

Table 2. Compounds, identified by gas chromatography linked mass spectrometry (GC-MS), recovered from rumen digesta headspace that evoked responses from *Stomoxys calcitrans* antennae during GC electroantennographic detector (EAD) analysis (Fig. 1) on the polar column (see Materials and methods).

Compound name	%	RI(1)	RI(2)	EAD response *n*°	EAD (mV)
Acetic acid	12.4	1461	1457	12	1.16
Propanoic acid*	13.5	1541	1541	14	1.15
Isobutyric acid*	8	1567	1568	17	0.32
Butanoic acid*	13.6	1622	1625	19	1.04
Isovaleric acid*	12.3	1667	1669	21	0.25
Hexanoic acid*	6	1839	1841	24	0.57
Octan-3-ol	2	1390	1388	9	0.64
(*Z*)-hex-3-en-1-ol*	2	1393	1391	9	0.64
Oct-1-en-3-ol*	0.1	1449	1448	11	1.19
Heptan-1-ol*	12.4	1458	1457	12	1.16
Octan-1-ol*	0.7	1556	1558	15	0.55
Acetophenone*	0.2	1665	1660	20	0.75
Octan-3-one*	5.6	1248	1244	4	0.69
6-methylhept-5-en-2-one*	0.7	1338	1347	5	0.61
Undecan-2-one*	13.5	1538	1543	14	1.15
Skatole*	1.4	2498	2495	26	0.31
p-cresol*	5.6	2085	2085	25	0.54
Dimethyl trisulphide*	0.7	1361	1377	6	1.02
Citronellene*	8.9	1028	1056	1	0.47
D-limonene*	3.6	1180	1193	3	0.37
β-cyclocitral*	0.5	1607	1598	18	1.08
β-caryophyllene*	0.1	1564	1590	16	0.45
1-methyl-3-(1-methylethyl)-cyclohexene	3.3	1143	–	2	0.58
1-propenylbenzene	0.1	1384	–	7	0.39
2,3,6-trimethylhepta-1,5-diene	Tr	1386	–	8	0.87
Unidentified	0.1	1414	–	10	0.38
Unidentified	0.3	1516	–	13	0.75
Unidentified	0.2	1715	–	22	0.58
Unidentified	0.1	1763	–	23	0.58

*Synthetic analogue showed electrophysiological activity in GC-EAD analyses or EAG recordings from *S. calcitrans.*
RI(1), retention index of natural product.
RI(2) retention index of injected synthetic analogue in gas chromatography linked mass spectrometry analyses or RI from the literature (www.flavornet.org).
Tr, a product present at $< 0.1\%$ in the extract.

Electroantennogram and GC-EAD recordings with synthetic chemicals

Electroantennogram responses of *S. calcitrans* were recorded to different doses of synthetic analogues of some EAD-active constituents of the rumen volatile extract (Figs 2 and 3). These recordings indicate oct-1-en-3-ol elicits the strongest response throughout the dose range tested (Fig. 2). Electroantennogram responses to this substance were discernible with the delivery of approximately 100 femtograms to the antennal preparation in the current set-up. Likewise, stable fly antennae responded to dimethyl trisulphide at a threshold in the hundreds of femtogram range and this compound elicited the second highest EAG response amplitudes (Fig. 2). Electroantennogram responses to β-caryophyllene, α-humulene and decanal were discernible at the lowest dose (10 ng on the filter paper), suggesting a sensory threshold in the picogram range for these compounds (Fig. 3). Butanoic acid and *p*-cresol evoked EAG responses at doses between 100 ng and 1 μg in the stimulus syringe, indicating a threshold close to 10 pg. The response threshold to skatole was the highest among the compounds tested (Fig. 2).

The EAD responses of a female antenna to the same amount of six products (i.e. dimethyl trisulphide, oct-1-en-3-ol, butanoic acid, isovaleric acid, *p*-cresol and skatole) at increasing doses confirmed the EAG assays (Fig. 4). These tests revealed that *S. calcitrans* antennal receptor cells exhibit the lowest sensory threshold for dimethyl trisulphide among the compounds tested. We noted a stronger antennal response to this product compared with oct-1-en-3-ol at the lowest dose; corresponding to, at most, 100 femtograms reaching the antenna. An EAD response was also recorded to *p*-cresol at 20 ng, corresponding to a sensory threshold in the picogram range. The thresholds recorded for butanoic and isovaleric acids were found to be slightly higher than for *p*-cresol, at 1−5 pg. No significant EAD response was recorded to skatole at the highest dose (100 ng), indicating a threshold for this product between 10 pg and 100 pg (Fig. 4).

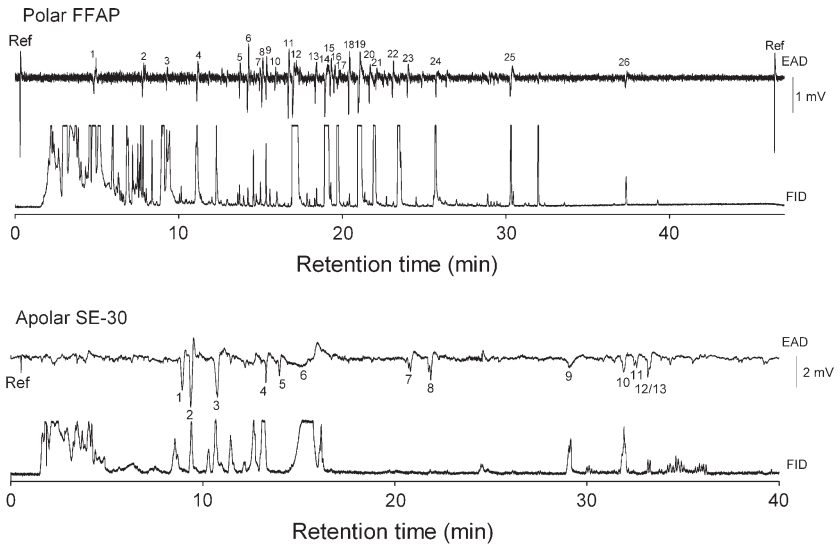

Fig. 1. Representative gas chromatographic profiles of fresh rumen volatiles on polar (FFAP) and apolar (SE-30) phases and associated electroantennogram recordings from *Stomoxys calcitrans*. The lower trace in each case is the flame ionization detector (FID) response and the upper trace the electroantennographic detector (EAD) response of *S. calcitrans* to the biologically active constituents of the rumen odour extract. The identity of numbered EAD active constituents was confirmed by gas chromatography linked mass spectrometry (see Tables 1 and 2 for details). Reference responses (Ref) were recorded at the start and end of analysis using 1 μg oct-1-en-3-ol in a stimulus syringe.

Wind tunnel experiments

Of the stable flies tested, 66% were activated by rumen digesta and 37% by the control. However, activated flies showed a low propensity (14%) for flight towards the source after leaving the release cage (Table 3), although they did respond to rumen volatiles, showing a significantly higher rate of orientated flights than controls (Table 3). A 20 ± 10 p.p.m. increase in the level of CO_2 was measured at the rumen digesta odour delivery point in the wind tunnel, but dilution in the wind tunnel resulted in no variation in CO_2 level at the insect release point.

Among rumen volatiles tested, dimethyl trisulphide, butanoic acid and *p*-cresol activated and attracted flies in a manner significantly different from the control (Table 4). Dimethyl trisulphide, the most active compound, activated 73% of the flies, and attracted 41%, giving an average of 2.2 ± 0.2 orientated flights/fly. Butanoic acid activated 68% of flies with 21% showing attraction and making an average of 1.5 ± 0.2 orientated flights/fly. Similarly, *p*-cresol activated 75% of flies and attracted 21%, giving a mean of 1.3 ± 0.2 orientated flights/fly. Responses to oct-1-en-3-ol and skatole did not differ from those to the control (Table 4). However, a significantly higher number of orientated flights (1.3 ± 0.3) were recorded during exposure to oct-1-en-3-ol.

Presenting oct-1-en-3-ol and dimethyl trisulphide together, each at 10 μg in the dispenser, did not increase activation (70%), attraction (21%) or the number of orientated flights (1.8 ± 0.3) compared with dimethyl trisulphide on its own. The percentage of activated and attracted flies, and the number of orientated flights recorded for this binary mixture was, nevertheless, significantly different from those for the control. A higher level of activation (84%) was recorded in response to a second mixture, composed of dimethyl trisulphide, butanoic acid and *p*-cresol

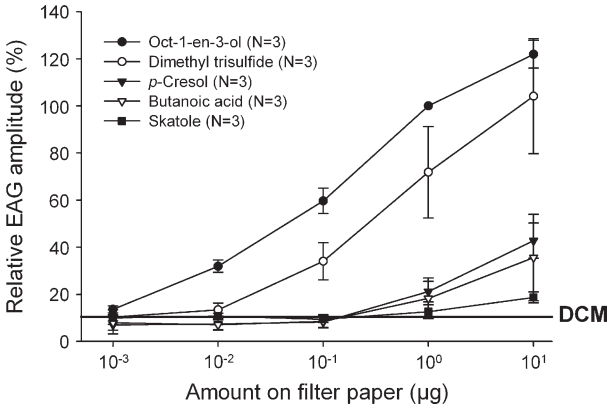

Fig. 2. Electroantennogram responses (± standard error) of *Stomoxys calcitrans* antennae ($n = 3$) to increasing doses (10^{-3} μg to 10^{1} μg) of five chemostimuli identified in rumen fluid odour. Responses are normalized using 1 μg oct-1-en-3-ol as reference (100%). The response to methylene chloride (DCM, solvent control) provides an estimation of the response threshold for each compound. EAG, electroantennogram.

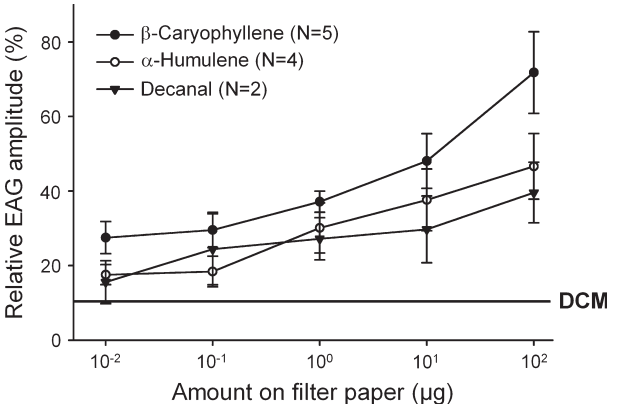

Fig. 3. Electroantennogram responses (± standard error) of *Stomoxys calcitrans* antennae ($n \geq 2$ and ≤ 5) to increasing doses (10^{-2} μg to 10^{2} μg) of three chemostimuli identified in rumen fluid odour. Responses are normalized using 1 μg oct-1-en-3-ol as reference (100%). The response to methylene chloride (DCM, solvent control) provides an estimation of the response threshold for each compound. EAG, electroantennogram.

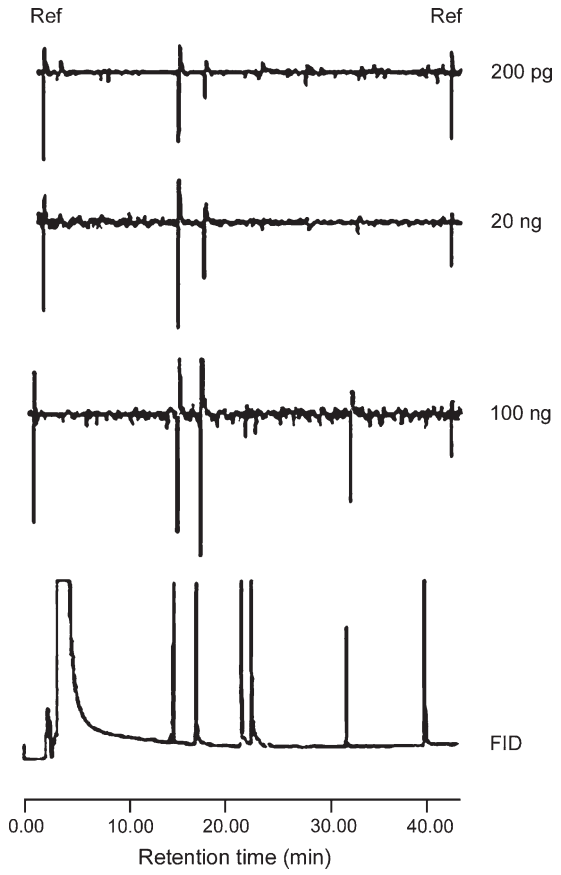

Fig. 4. Representative electroantennographic detector responses of a female *Stomoxys calcitrans* antenna to similar amounts of six rumen volatiles injected at successively higher doses on to a gas chromatographic column (FFAP). The lowest trace is the flame ionization detector (FID) response to the six products listed in order of elution, from left to right: dimethyl trisulphide, oct-1-en-3-ol, butanoic acid, isovaleric acid, *p*-cresol and skatole. Reference responses (Ref) were recorded at the start and end of each analysis using 100 ng oct-1-en-3-ol in a stimulus syringe.

each at 10 μg in a dispenser, but attraction (29%) and orientated flights were lower than with dimethyl trisulphide alone. Nevertheless, the rate of attraction and the number of orientated flights recorded to mixture 2 were higher than those to either butanoic acid or *p*-cresol presented alone at the same dose (Table 4).

Discussion

We found that rumen digesta activated stable flies and induced a significantly higher number of orientated flights in the plume than did the control. Moreover, the EAD data show that *S. calcitrans* antennal receptor cells are sensitive to a range of rumen volatile constituents. The percentages of activated and attracted *S. calcitrans* observed in the wind tunnel with dimethyl trisulphide, butanoic acid and *p*-cresol either presented individually or in mixtures confirms that several rumen volatiles attract stable flies. The presence in bovine breath of volatile organic compounds, such as propionic acid, isovaleric acid, hexanoic acid, decanal and acetophenone (Spinhirne *et al.*, 2004), also found as chemostimuli for *S. calcitrans* in our analyses of rumen odour extracts, underlines the importance of rumen chemostimuli in host location by these flies.

Dimethyl trisulphide from rumen volatiles was identified as a stable fly chemostimulant, and, despite being present in only low amounts, elicited strong and consistent EAD responses. A low response threshold to dimethyl trisulphide was confirmed from the EAG dose–response curves and the GC-EAD recordings, both of which showed sensitivity below the 10^2-femtogram threshold. The high volatility of this product may partly explain the slightly higher threshold found in EAG recordings compared with GC-EAD analyses. Dimethyl trisulphide, like dimethyl disulphide, arises from the oxidation of methanethiol, derived from methionine (Weimer *et al.*, 1999) and has been identified in cheese flavour (van Kranenburg *et al.*, 2002), human skin

Table 3. Behavioural responses of *Stomoxys calcitrans* in a wind tunnel to rumen digesta and to the control (distilled water). Oriented flights in the odour plume are the mean number per activated fly (± standard error).

	Control	Rumen
% of flies activated	37.1	65.7†
% of flies attracted	7.1	14.3
Oriented flights in plume	0.6 ± 0.2	1.5 ± 0.2*

*$P < 0.01$; †$P < 0.001$ compared with the control. Chi-square tests were performed on counts of activation and attraction. Mann–Whitney test was used to test for significance in the number of orientated flights; 70 flies were tested per treatment.

Table 4. Behavioural responses of *Stomoxys calcitrans* to rumen volatiles in a wind tunnel. Treatments consisted of 10 μg test substances in a polyethylene vial. The control was methylene chloride (DCM). Orientated flights in the plume are the mean number per activated fly (± standard error).

	% of flies activated	% of flies attracted	Orientated flights in plume
Control (DCM)	48.8	7.5	0.7 ± 0.1
Oct-1-en-3-ol	57.5	17.5	1.3 ± 0.3*
Dimethyl trisulphide	72.5†	41.3‡	2.2 ± 0.2‡
p-cresol	75.0‡	21.25*	1.3 ± 0.2*
Butanoic acid	67.5*	21.3*	1.5 ± 0.2‡
Skatole	57.5	13.75	0.9 ± 0.2
Mix 1§	70†	21.25*	1.8 ± 0.3‡
Mix 2§	83.8‡	28.8‡	1.5 ± 0.2†

*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ compared with the control.
Chi-square tests were performed on counts of activation and attraction. Mann–Whitney test was used to test for significance in the number orientated flights; 80 flies were tested per treatment.
§Mix 1 = dimethyl trisulphide and oct-1-en-3-ol, each at 10 μg in the dispenser; mix 2 = dimethyl trisulphide, butanoic acid and *p*-cresol, each at 10 μg in the dispenser.

emanations (Bernier *et al.*, 2000) and livestock wastes (Mackie *et al.*, 1998; Louhelainen *et al.*, 2001; Le *et al.*, 2004), and may even occur in human breath (Kaji *et al.*, 1978; Wahl *et al.*, 1996). As well as being present in a wide range of odour sources implicated in host location by *S. calcitrans*, dimethyl trisulphide serves as a strong attractant for some calliphorids and the muscid *Hydrotaea anxia* in the field (Nilssen *et al.*, 1996), and attracts house flies in wind tunnel assays when mixed with butanoic acid and skatole (Cossé & Baker, 1996). Our wind tunnel observations demonstrate that dimethyl trisulphide on its own attracts stable flies.

Gas chromatography MS analysis of the rumen odour extract confirmed the presence of large amounts of carboxylic acids, whose importance for host location by haematophagous arthropods has been widely demonstrated (Knols *et al.*, 1997; Bosch *et al.*, 2000; Guerenstein & Guerin, 2001; Donzé *et al.*, 2004). Our GC-EAD recordings show that *S. calcitrans* can perceive a range of straight and branched carboxylic acids and that butanoic acid, at a source dose of 10 μg, attracted stable flies in our wind tunnel assay. Consistent EAD responses were also recorded to *p*-cresol and skatole as constituents of rumen fluid that originate from tryptophan degradation by rumen bacteria (Mohammed *et al.*, 2003). In the wind tunnel, *p*-cresol elicited significant activation and attraction at the level of 10 μg in the dispenser. We suggest that, assuming a sensory threshold at around 10 pg, *p*-cresol is most probably used by stable flies for host location in the same way that *p*-cresol and 3-n-propyl phenol in cattle urine is used by tsetse flies (Bursell *et al.*, 1998).

Rumen digesta was also found to release various aliphatic alcohols and oct-1-en-3-ol was, surprisingly, detected in this anaerobic substrate, although this product arises from the enzymatic oxidation of linoleic acid. It may be that aerobic processes set in during rumen digesta collection and transport to the laboratory. Oct-1-en-3-ol was the compound to which *S. calcitrans* showed an antennal response, even at 1 ng on filter paper, confirming the results reported by Schofield *et al.* (1995). Hall *et al.* (1984) identified oct-1-en-3-ol as a tsetse fly attractant in cattle odour but field studies (Mullens *et al.*, 1995; Cilek, 1999) showed that oct-1-en-3-ol failed to attract stable flies to cylinder traps, and neither attraction nor orientation was recorded to oct-1-en-3-ol in a triple cage olfactometer (Alzogaray & Carlson, 2000). Our observations in the wind tunnel tend to corroborate the latter findings in that no clear activation or attraction was recorded upon exposure of *S. calcitrans* to oct-1-en-3-ol on its own, although we did observe a significantly higher number of orientated flights in the plume compared with the control. No additional effects were observed when oct-1-en-3-ol was mixed with dimethyl trisulphide. Such findings are surprising in view of the low threshold of *S. calcitrans* antennal receptor cells for this product. The most likely explanation is that the appropriate dose for eliciting activation or attraction was not tested, or that oct-1-en-3-ol only makes a contribution to mixtures of other host volatiles.

Terpenes were detected in GC-EAD analysis of rumen volatiles and the rumen digesta constituents citronellene, β-cyclocitral, D-limonene, dihydrocarvone, geranyl acetone, β-caryophyllene and α-humulene were found to elicit EAD responses from *S. calcitrans*. This is not surprising that stable flies (Jones *et al.*, 1992), like mosquitoes (Bowen, 1992), that are known to feed on nectar in nature should respond to these typical plant products. An antennal threshold in the picogram range by *S. calcitrans* for β-caryophyllene and α-humulene was recorded in this study, and even an obligate haematophagous insect such as the tsetse fly (Syed & Guerin, 2004) responds to terpenes. This suggests that these chemostimuli may also be used for host location.

It is also worth mentioning the detection in rumen volatiles of ketones, arising from different biosynthetic pathways, as chemostimulants for *S. calcitrans,* with acetophenone being derived from L-phenylalanine, octan-3-one and undecan-2-one from fatty acid degradation, and 6-methylhept-5-en-2-one as an isoprenoid derivative. Interestingly, acetophenone and 6-methylhept-5-en-2-one are commonly encountered in sources of volatiles known to attract haematophagous arthropods, such as human or bovine breath (Wahl *et al.*, 1996; Phillips *et al.*, 1999; Spinhirne *et al.*, 2004), bovine odour (Birkett *et al.*, 2004), human skin emanations (Bernier *et al.*, 2000) and human sweat (Meijerink *et al.*, 2000). Although not tested in our behavioural experiments, ketones may also be involved in host location by *Stomoxys*.

This study shows that *S. calcitrans* is extremely sensitive to some volatile constituents of rumen odour. Many of these compounds are not exclusive to the rumen but also occur in a range of other resources. Oviposition substrates exploited by gravid *Stomoxys* females (Jeanbourquin & Guerin, 2007) also release some of these products. Stable flies are probably able to make parsimonious use of products for locating hosts, nectar and oviposition substrates.

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