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ORIGINAL PAPER

Olfactory and behavioural responses of tsetse flies, *Glossina* spp., to rumen metabolites

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Abstract Herbivores provide tsetse flies with a blood meal, and both wild and domesticated ruminants dominate as hosts. As volatile metabolites from the rumen are regularly eructed with rumen gas, these products could serve tsetse flies during host searching. To test this, we first established that the odour of rumen fluid is attractive to hungry *Glossina pallidipes* in a wind tunnel. We then made antennogram recordings from three tsetse species (*G. pallidipes* morsitans group, *G. fuscipes* palpalis group and *G. brevipalpis* fusca group) coupled to gas chromatographic analysis of rumen fluid odour and of its acidic, mildly acidic and neutral fractions. This shows tsetse flies can detect terpenes, ketones, carboxylic acids, aliphatic aldehydes, sulphides, phenols and indoles from this biological substrate. A mixture of carboxylic acids at a ratio similar to that present in rumen fluid induced behavioural responses from *G. pallidipes* in the wind tunnel that were moderately better than the solvent control. The similarities in the sensory responses of the tsetse fly species to metabolites from ruminants demonstrated in this study testify to a contribution of habitat exploitation by these vertebrates in the Africa-wide distribution of tsetse.

Keywords Tsetse flies · Sensory physiology · Rumen metabolites · Olfaction · Behaviour

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Introduction

There are 23 species of tsetse flies (*Glossina* spp.; Diptera: Glossinidae) plus 8 subspecies currently confined to sub-Saharan Africa. Tsetse subgenera correspond to their three preferred habitats (Jordan 1986): morsitans flies occupying woodland savannah, palpalis flies in lowland rain forest along watercourses, and fusca flies inhabiting lowland rainforest and gallery forests. These three subgenera are represented in this study by, respectively, *G. pallidipes* (Austen), *G. fuscipes* (Newstead) and *G. brevipalpis* (Newstead). During their blood meal, tsetse flies can transmit protozoans of the genus *Trypanosoma* which can debilitate both man and his livestock. Tsetse fly locates hosts using vision and, beyond their visual range, by following olfactory cues (Vale 1977).

A variety of visual traps and targets have been developed for tsetse flies providing an ecologically viable and efficient method to eradicate populations of these disease vectors (Vale et al. 1988) and to prevent the reinvasion of tsetse free zones (Politzar and Cuisance 1983; Muzari 1999). Trap efficiency can be improved by the addition of products from host animals such as CO_2 (Rennison and Robertson 1960), acetone (Vale 1980), 1-octen-3-ol (Hall et al. 1984) and phenols identified from cow urine (Owaga 1985; Bursell et al. 1988).

A combination of 3-*n*-propyl phenol, 1-octen-3-ol, *p*-cresol and acetone significantly augments trap catches of tsetse flies of the morsitans group but not catches of palpalis and fusca spp. (Green 1994; Mwangelwa et al. 1995), even though some species like *G. tachinoides* and *G. brevipalpis* in these tsetse fly groups respond well to some mixtures. However, since the initial isolation of semiochemicals for tsetse, little research on molecules influencing tsetse fly sensory ecology has been undertaken,

even though field experiments had shown that tsetse flies are able to react to a larger variety of chemostimuli (Vale 1980; Torr et al. 1996; IAEA-TECDOC 2003). More recently, laboratory experiments have shown that tsetse flies of the three subgenera are attracted to plant compounds in a wind tunnel (Syed and Guerin 2004).

Herbivores are among the preferred hosts for tsetse flies due to their habit of herding and their sessile nature and the known tsetse fly attractants 3-*n*-propyl phenol, 1-octen-3-ol, *p*-cresol and acetone emanate from these animals. Ruminants form a major group of wild and domesticated mammals that are present in all tsetse fly habitats as their diversification permits them to exploit various types of plants. In the first chamber of their forestomach, the rumen, a variety of micro-organisms hydrolyse plant tissues to yield energy. This process inside a stable bioreactor leads to the formation of products which do not vary greatly between ruminant species (Clarke and Bauchop 1977). As these products formed in the foregut are continually evacuated through eructation, they may provide tsetse flies with important cues to track ruminants across the range of habitats they have come to occupy in sub-Saharan Africa. Moreover, rumen fluid odour has already been found to attract other haematophagous arthropods linked to large mammalian herbivores, namely the adult stage of five hard tick species that take large blood meals on such hosts (Donzé et al. 2004) and the stable fly *Stomoxys calcitrans* that is a worldwide pest of livestock (Jeanbourquin and Guerin 2007). Considering the success of tsetse flies in exploiting herbivore mammals, especially ruminants, we hypothesised that volatiles from the rumen might serve as a source of chemostimuli for tsetse species of different subgenera.

Methods and materials

Insects

Pupae were obtained from the International Atomic Energy Agency (IAEA, Vienna). Adult *G. pallidipes*, *G. fuscipes* and *G. brevipalpis* were maintained in rectangular cotton netting cages (1-mm mesh, 25 × 15 × 15 cm). They were covered by a transparent plastic bag containing a wet tissue to maintain 100% RH in an environmental chamber at 26°C with 8-h light and 22°C with 10-h dark, with 2-h light ramps at dawn and dusk. Unmated flies were sexed just after emergence (3 days max) and fed on manually defibrinated bovine blood at 2- to 3-day intervals from day 2 after emergence. Flies used for electrophysiological recordings were unmated, unfed and only 2–4 days old to assure viable antennal preparations. Flies tested in the wind tunnel had at least two blood meals to permit them to develop their flight muscles (Langley 1970), they were then starved for 3–5 days and held during experiments in the environmental chamber (25°C, 65% RH) housing the wind tunnel (see below).

Rumen fluid collection and fractionation

Rumen fluid was collected by squeezing the bolus from freshly slaughtered cattle at an abattoir (La Chaux-de-Fonds, CH). The cattle were slaughtered at different times of the year and fed silage and hay during winter and fresh grass during summer. Headspace collection of the rumen fluid volatiles and chemical fractionation of the bolus was performed on the day of collection. For headspace collection, rumen volatiles were trapped by passing N_2 for 3 h at 50 ml min^{-1} through a 1-l desiccator maintained at 35°C connected to a Pasteur pipette (5 mm i.d.) containing 500 mg preconditioned PorapakQ® (60–80 mesh, Millipore Corporation, USA) and adsorbed volatiles were desorbed with 500 µl of dichloromethane (DCM). For rumen fluid fractionation, after centrifugation (30 min at 3,600 rpm) the rumen supernatant was brought from pH 5–6 to pH 14 with 10% NaOH. At this pH amines and neutral molecules were extracted into dichloromethane (DCM, Merck, analytical grade) and termed the neutral fraction in the text. The rumen fluid was then brought to pH 0 with 20% H_2SO_4 and the remaining organic compounds extracted into DCM. This organic fraction in DCM was then washed with $NaHCO_3$ solution (pH 9) so that phenols in the nonionised state were retained in the organic phase, termed the mildly acid fraction, whereas carboxylic acids were recovered in the aqueous phase. These acids were subsequently extracted with DCM after acidification as above, giving the acid fraction.

Electroantennogram recording

The electroantennogram (EAG) permits measurement of the electrophysiological responses of insect antennal receptor cells on stimulation with an odour (Schneider 1957). EAGs from tsetse antennae were recorded as described in Syed and Guerin (2004).

Gas chromatography linked electroantennogram recording

Coupling the EAG as a biological detector to the effluent of a high-resolution gas chromatographic (GC) column permitted the localisation of the biologically active components of rumen fluid and its fractions (GC-EAG; Arn et al. 1975). High-resolution gas chromatographic separations were made in a gas chromatograph (5300, Carlo Erba Instruments, Italy) on a polar free fatty acid phase (30 m, 0.250 mm i.d., BGB, CH). In order to compensate

the differences in sensitivity of antennae between different analyses and insects, the antennal preparation was subjected to an odour pulse from 1 µg of 1-octen-3-ol (50:50 R/S racemic mixture, >97% pure, Merck) in a 5-ml plastic syringe at the start and end of each GC run. All the EAG responses recorded during a GC run were normalised using the mean EAG responses to these two odour pulses of 1-octen-3-ol.

Chemicals

The synthetic compounds used for GC-EAG recordings and behavioural experiments included: sulphides (dimethyl disulphide, dimethyl trisulphide) and carboxylic acids (unbranched C_2–C_6, isobutanoic, 2-methyl butanoic, isovaleric, 2-methyl pentanoic and cyclohexane carboxylic acid) all ≥97% pure. Terpenes tested were iso-caryophyllene, β-farnesene and germacrene D present in a mixture of various terpenes (Firmenich, CH). Identity of biologically active compounds in the rumen extract was determined by GC-MS in a Hewlett Packard 5890 series II chromatograph (column and conditions as in GC-EAD) with a mass selective detector (HP 5971A). Compounds found in samples of the rumen odour extracts and fractions (see above) were identified by comparing the mass spectra of unknowns to those of standards in a mass spectrum library (Nist98) and by comparing the Kovats retention indices of unknowns with those of standards (Table 1).

Behavioural experiments

A wind tunnel (170 cm long, 60 × 60 cm) constructed of non-reflecting glass was used to test tsetse fly behavioural responses to test stimuli. Two centrifugal ventilators at either end of the wind tunnel operated simultaneously to move air across its length at 30 cm s^{-1} through active charcoal cartridges and semi-laminar perforated screens (1-mm-thick steel plate with 3-mm round holes, 51% air passage). Overhead illumination was provided by high-frequency fluorescent lights (36 W, >1 kHz, Philips) running the length of the tunnel (~300 lux on the floor). The wind tunnel sides were rendered uniformly white with white cotton curtains. The structure of the plume of odour was visualized by generating a plume of ammonium acetate.

Two different setups were used for the behavioural experiments: the method already described in Syed and Guerin (2004) was used to test the responses of *G. pallidipes* to the odour of the rumen fluid, whereas the method explained below was employed to test the responses of *G. pallidipes* and *G. brevipalpis* to known compounds.

For tests with known compounds, a brown paper sheet was placed on the floor with a pale blue strip of paper (6.5 cm wide) on top running the length of the wind tunnel in the middle of the floor. The tunnel ceiling was covered with a white sheet of paper with 35 black rectangular patches (4 × 8 cm) fixed at random to serve as an optomotor cue. Flies were transferred individually into a plastic release cage (transparent 15 cm long, 10 cm diameter, PVC cylinders) with both ends covered with nylon netting (1-mm mesh) doors. The base of the release cage was placed horizontally at a height of 30 cm at 10 cm from the downwind end of the tunnel and after 1 min of acclimatisation both doors were lifted slowly using pulleys. If the fly did not exit during cage, it was successively exposed for 1 min to odour-free air and then for 1 min to the test product. Effects of test stimuli were quantified by recording either 3 or 4 of

Table 1 Kovats retention indices (KI) for EAG-active products in rumen fluid headspace vapour and in rumen fluid fractions eluting on a free fatty acid phase compared to KI of standard compounds analysed on the same chromatographic phase (see "Materials and methods")

	Kovats retention indices		
	EAG-active product in rumen fluid fractions	EAG-active product in rumen fluid fractions	Standard compound[a]
Tricyclene	1002		–
α-Pinene	1027		1007
Camphene	1074		1068
β-Pinene	1110		1138
Limonene	1188		1191
2-Heptanol	1336		–
2-Octanol	1398		1352
Acetic acid	1457	1449	1447
Benzaldehyde	1502		1525
Camphor	1507		–
Propanoic acid	1541	1534	1534
Isobutanoic acid	1571	1564	1570
β-Caryophyllene	1575		1590
Butanoic acid	1625	1620	1623
Isovaleric acid	1662	1662	1670
β-Selinene	1714		1781
Pentanoic acid	1733	1738	1745
Hexanoic acid	1841	1836	1835
Heptanoic acid		1928	1924
6-Methyl 2-heptanone	2035		–
Cyclohexane carboxylic acid		2050	2053
p-Cresol	2092	2080	2083
Indole	2455		2455
Skatole	2510		2495

[a] In addition to their identification by comparison of their mass spectra with the mass spectra library, retention indices of injected standard compounds in gas chromatography equipped with FFAP column were measured when possible (for more detail see "Materials and methods")

the following behaviour elements: *activation* when the fly moved in the cage, *exit* when the fly flew from the cage, *directed flight* when the fly travelled at least 50 cm within the plume of odour, *attraction* when the fly made a directed flight to within 10 cm of the test stimulus source. Differences in behavioural criteria between treatments were calculated using Fisher's exact test in R software.

Test stimuli were introduced via an upright aluminium tube (7 mm i.d.) through the tunnel floor at 25 cm from the upwind end of the tunnel. This tube was bent downwind at a height of 40 cm and entered a plume generator that consisted of a stainless steel cylinder (10.5 cm long, 11 cm o.d.) with an aluminium tapered end (4 cm long, 11–14 cm o.d.) ending in a bronze grid (1-mm mesh). The plume generator and the semi-laminar flow screens produced an odour plume ca. 10 cm in diameter at 30 cm from the upwind end of the wind tunnel expanding to ~50 cm in diameter at 5 cm from the downwind end. A stimulus controller (CS-55, Syntech, NL) switched the charcoal-filtered airflow between test and control flows for the successive 1 min control and test periods.

For experiments to record responses of *G. pallidipes* ($n = 50$) to the carboxylic acid mixtures, odour-free air at 0.5 l min^{-1} passed through a 1-l gas-wash bottle in which 100 µl of the test mixture was applied on a filter paper disk (diameter 9 cm). Carboxylic acid mixtures tested were a blend of butanoic, pentanoic, hexanoic and isovaleric acids diluted at 100 ng/µl in DCM at 2 different ratios: an equimolar 1:1:1:1 mixture or a 4:1:1:1 mixture mimicking the ratios at which the acids occur in rumen fluid (see "Results"). Negative controls for this experiment consisted of charcoal-filtered air passing at 0.5 l min^{-1} through a 1-l gas-wash bottle containing a filter paper disk treated with 100 µl of pure DCM or an empty 1-l gas-wash bottle. Fifty flies were tested with each treatment.

For experiments with acetone added to the carboxylic acids, *G. brevipalpis* ($n = 40$ with 50/50 females/males) were tested for each treatment using two air streams. One stream of charcoal-filtered air at 5 ml min^{-1} passed through a 1-l gas-wash bottle containing a polyethylene sachet (4 × 5.5 cm, 150 µm wall thickness) loaded with 1 ml of either isopropanoic, butanoic, isovaleric, valeric, hexanoic or cyclohexane carboxylic acid. A second stream of charcoal-filtered air at 500 ml min^{-1} passed through the second 1-l gas-wash bottle containing 5 ml of pure acetone (99.8% pure, Acros, USA). The two streams were mixed before entering the upright aluminium tube leading to the plume generator. The release rate of the carboxylic acids from the polyethylene sachet was calculated by measuring the weight loss more than 20 times over 4 days (AE100 balance, Mettler-Toledo, Switzerland) and for acetone by weighing the bottle containing acetone before and after experiments. With a plume volume of 120 l/min, we could estimate the amount of each compound inside the plume at 2.67 µg/l acetone, 0.26 µg/l isobutanoic acid, 0.51 µg/l butanoic acid, 0.19 µg/l isobutanoic acid, 0.38 µg/l pentanoic acid, 0.29 µg/l hexanoic acid and 0.06 µg/l cyclohexane carboxylic acid. The same method was used to test *G. brevipalpis* responses to the mixture of 3-*n*-propyl phenol, 1-octen-3-ol and *p*-cresol at a ratio 1:4:8 dispensed from a polyethylene sachet (as described above) on its own and with acetone. For the negative control, both 1-l gas-wash bottles were empty.

Results

Tsetse behavioural and EAG responses to rumen fluid odour constituents

Rumen fluid odour attracted *G. pallidipes*: 57% of the 75 flies tested were activated upon exposure to the odour, and 72% of these activated flies reached the source. The blank air control elicited no response. Activation occurred within 30 s of odour delivery and flies reaching the source did so within 60 s of exposure. Flies typically showed short flights of 0.2–0.5 m to reach to the source, correcting to reorientate upwind on the cylinder walls before each take off (2-3 take offs per fly).

The antennae of *G. brevipalpis*, *G. fuscipes* and *G. pallidipes* responded to compounds of different chemical classes in the rumen fluid odour extract as analysed by GC-EAG (Fig. 1). The main aromatic chemostimulants, 4-methyl-phenol (*p*-cresol), indole and skatole activated the antennae of the three tsetse fly spp. Other rumen odour constituents that elicited EAG responses were 2-heptanol, 2-octanal and 6-methyl 2-heptanone and the sesquiterpenes *β*-caryophyllene and *β*-selinene. *G. brevipalpis* antennae also responded to the early eluting monoterpenes tricyclene, α-pinene, camphene, and *β*-pinene. The most conspicuous aspect of the EAG response profiles is the strong degree of similarity in the responses of the three species to the main carboxylic acid rumen chemostimulants. Butanoic, isobutanoic, pentanoic and hexanoic acid gave the strongest responses, but responses to acetic, propanoic and isopropanoic acid were also recorded.

Tsetse fly EAG responses to constituents of the mildly acidic fraction of rumen fluid

In this fraction, which in principle permits isolation of the mildly acidic phenols and indoles, *p*-cresol predominated in the four separate mildly acidic fractions extracted from three rumen fluid samples as analysed by GC-MS. Only *p*-cresol (present at >250 µg as extracted from 15 ml of rumen fluid) elicited a good EAG response from

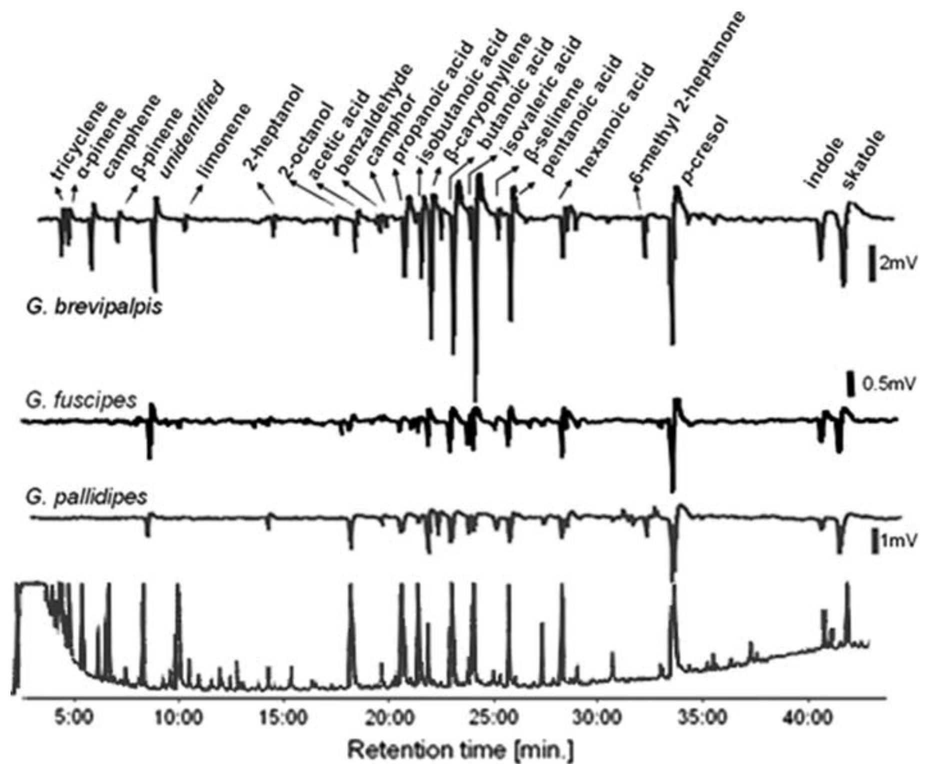

Fig. 1 Electroantennogram responses of the tsetse flies *G. brevipalpis*, *G. fuscipes* and *G. pallidipes* (*from top to bottom*) to rumen fluid odour constituents collected on PorapakQ® polymer and fractionated by gas chromatography. The lower trace is the flame ionisation detector response and the upper three traces are the electroantennogram responses of the three species to the biologically active constituents of the odour extract. *Scale bars* in mV (different for each species) provide a measure of the response amplitudes recorded

G. pallidipes and *G. brevipalpis* (not shown). Other aromatic products present at small amounts (yield <15 µg as extracted from 15 ml of rumen fluid) in this fraction did not elicit any EAG responses from the two species tested.

Tsetse fly behavioural and EAG responses to constituents of the acidic fraction of rumen fluid

Propanoic, butanoic, valeric and hexanoic acid and their branched isomers, i.e. isobutanoic and isovaleric acids were major volatile constituents of the acidic fraction from rumen bolus (Fig. 2). The relative composition and quantity of carboxylic acids was almost identical in 6 separate analyses of 5-10 µl aliquots of the acidic fraction from 3 different rumen fluid collections. Butanoic acid was always the predominant carboxylic acid yielding on average >2 mg from 15 ml of rumen fluid, with propanoic, isovaleric, valeric and hexanoic acid present at amounts some 25% of the major constituent. All the other carboxylic acids were present at low amounts (yields less than 10% that of butanoic acid) except benzenepropanoic acid for which yields were inconsistent. Overall, the variability in the amounts of the aromatic carboxylic acids was much higher.

The EAG response profiles of *G. pallidipes*, *G. fuscipes* and *G. brevipalpis* were similar to the major volatile components of rumen fluid, i.e. the carboxylic acids detected in both the rumen headspace vapour collection and the acidic fraction of the fluid. All three tsetse fly species showed EAG responses to the unbranched C_2–C_8 carboxylic acids (Figs. 1, 2, 3), to the two branched carboxylic acids isobutanoic and isovaleric acid present in the rumen headspace vapour collection and the acidic extract (Figs. 1, 2), and to cyclohexane carboxylic acid in the acidic fraction (Fig. 2). The strongest EAG response recorded was to cyclohexane carboxylic acid relative to the lower level of this product in the fraction compared to the other carboxylic acids. The EAG response amplitudes recorded from the three tsetse species for the branched and unbranched carboxylic acids propanoic, isobutanoic, butanoic, isovaleric, valeric, hexanoic and heptanoic acids were approximately according to the amounts of these products present in the acidic fraction. This is to be expected considering how unbranched C_2-C_8 carboxylic acids evoke similar EAG response amplitudes from the three species (Fig. 3). The aromatic carboxylic acids, benzoic, benzene acetic and benzene propanoic acids present in the acidic fraction at relatively high amounts did not elicit EAG responses from any of the three tsetse fly species (Fig. 2).

To compare the sensitivity of tsetse flies to the same doses of carboxylic acids delivered to the antenna, EAG responses were recorded by injecting known amounts of unbranched acids onto a gas chromatographic column (Fig. 3). Unbranched carboxylic acids of C_4-C_7 chain length elicited higher EAG responses than those of lower or higher chain lengths across all the three species tested.

Behaviour responses of *G. pallidipes* were recorded in the wind tunnel to a mixture of four of the predominant carboxylic acids of rumen fluid that elicited EAG responses,

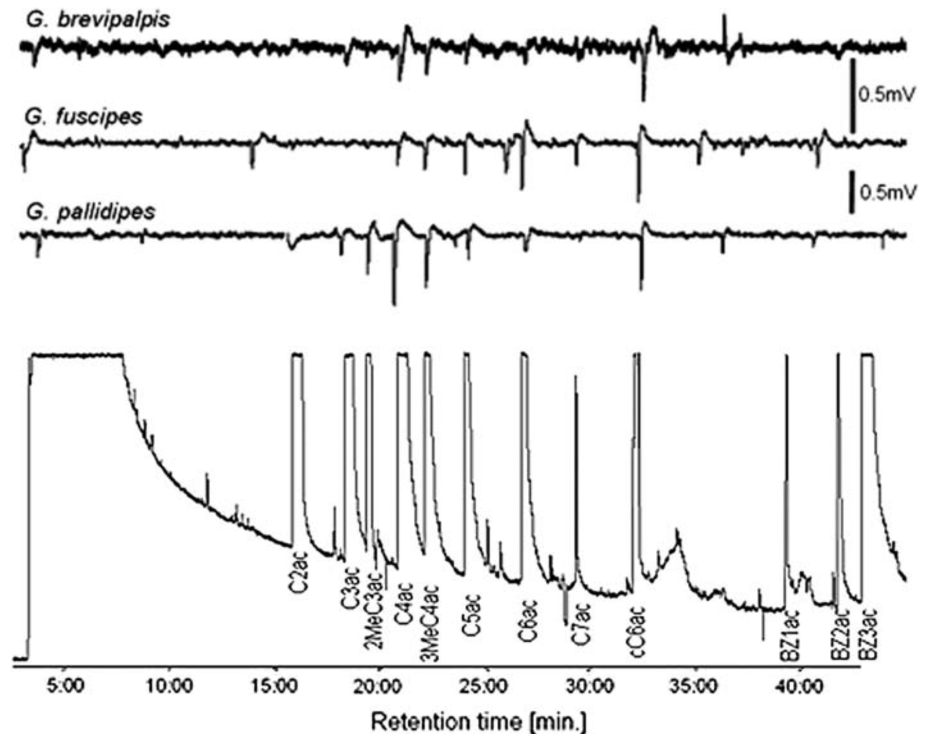

Fig. 2 Electroantennogram responses of the tsetse flies *G. brevipalpis*, *G. fuscipes* and *G. pallidipes* to constituents of the acidic fraction of rumen fluid, i.e. ethanoic (*C2ac*), propanoic (*C3ac*), isobutanoic (*2MeC3ac*), butanoic (*C4ac*), isovaleric (*3MeC4ac*), valeric (*C5ac*), hexanoic (*C6ac*), heptanoic (*C7ac*), cyclohexane carboxylic acid (*cC6ac*), benzoic (*BZ1ac*), benzenacetic (*BZ2ac*) and benzenepropanoic (*BZ3ac*) acids as they eluted from a gas chromatographic column. *Scale bars* in mV (common between *G. fuscipes* and *G. pallidipes*) provide a measure of the depolarisations recorded. For further details see legend to Fig. 1

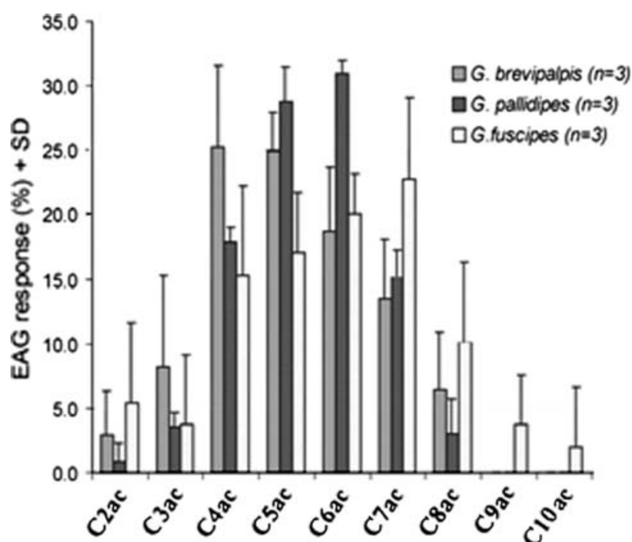

Fig. 3 Electroantennogram responses of the tsetse fly species *G. brevipalpis*, *G. pallidipes* and *G. fuscipes* to the unbranched acids ethanoic acid (*C2ac*) to decanoic acid (*C10ac*) delivered to the antennal preparations from a gas chromatographic column. A mixture containing 10 ng of each synthetic compound was injected and half of this amount was delivered to antenna after 50:50 split at the end of column length. Values are the percentage contribution of each acid to the pooled amplitude generated by all the biologically active acids in the mixtures and the standard deviation

namely butanoic, isovaleric, valeric and hexanoic acids. An equimolar mixture of these four acids did cause an increase in the level of activation of the flies in the release cage (18% of the 50 flies tested) compared to the blank control (4.7% of the 150 flies tested; $P < 0.05$), but this mixture failed to elicit a significant increase in cage exit, with 8 flies of 50 tested exiting the cage for the equimolar mixture compared to 6 flies of the 150 tested with the blank control ($P > 0.05$). However, a mixture of these four products presented at the ratio found in the acidic fraction of rumen fluid induced a higher level of activation (28% of the 50 flies tested), release cage exit (18% of the 50 flies tested) and attraction (10% of the 50 flies tested) compared to the equimolar mixture and to the blank control (with, respectively, 2% of the 50 and 150 tested flies attracted to the source). Even though the rumen-like mixture of 4 carboxylic acids elicited a stronger behavioural response, this was not significantly different from the responses induced by the equimolar carboxylic acid mixture nor the solvent control.

Since acetone is one of the major constituents of cow breath (Spinhirne et al. 2004) and known to enhance trap catches of tsetse flies when combined with other chemostimuli, the behavioural responses of *G. brevipalpis* were recorded in the wind tunnel to acetone presented alone as well as to mixtures of acetone with single carboxylic acids. Acetone alone caused a significant increase in the number of flies activated, exiting the release cage, making directed flights and arriving at the source over the blank control (air alone with no response; Table 2). Isobutanoic, butanoic, isovaleric, pentanoic, hexanoic and cyclohexane carboxylic acid presented alone did not induce a behaviour responses under any of the behavioural criteria recorded (data not shown). Adding any one of these carboxylic acids to acetone did not

Table 2 Behavioural responses of *G. brevipalpis* in the wind tunnel to acetone alone, to mixtures of acetone plus single carboxylic acid chemostimulants detected in rumen fluid odour, and to a ternary mixture of 3-*n*-propyl phenol, 1-octen-3-ol and *p*-cresol accompanied or not by acetone[a]

	n	Activation (%)	Exit the release cage (%)	Directed flight (%)	Attraction (%)
A	40	37.5***	30.0***	22.5**	20.0**
A + 2MeC3ac	40	42.5***	42.5***	20.0**	12.5
A + C4ac	40	27.5***	25.0**	15.0*	15.0*
A + 3MeC4ac	40	32.5***	27.5***	15.0*	10.0
A + C5ac	40	37.5***	30.0***	17.5*	12.5
A + C6ac	40	37.5***	32.5***	27.5***	7.5
A + cC6ac	40	47.5***	42.5***	30.0***	15.0*
POC	40	2.5	2.5	0	0
POC + A	40	35***	35***	27.5***	22.5**

[a] Tests with *G. brevipalpis* to acetone (*A*), carboxylic acids detected in rumen fluid odour, i.e. isobutanoic acid (*2MeC3ac*), butanoic acid (*C4ac*), isovaleric acid (*3MeC4ac*), valeric acid (*C5ac*), hexanoic acid (*C6ac*), cyclohexane carboxylic acid (*cC6ac*) and a mixture of 3-*n*-propyl phenol, 1-octen-3-ol and *p*-cresol (POC) at a ratio 1:4:8 released from a polyethylene sachet

Asterisks indicate that the percentage response is significantly different from that of the blank control at * $P < 0.05$; ** 0.01; *** 0.001 levels of probability using Fisher's exact test

The release rate of acetone presented neat in a gas-wash bottle and of different carboxylic acids as dispersed from polyethylene sachet was estimated in the plume to 2.67 µg/l A, 0.26 µg/l 2MeC3ac, 0.51 µg/l C4ac, 0.19 µg/l 3MeC4ac, 0.38 µg/l C5ac, 0.29 µg/l C6ac and 0.06 µg/l cC6ac

cause a significant increase or decrease in activation, release cage exit and attraction relative to that recorded for acetone alone ($P > 0.05$). Cyclohexane carboxylic acid plus acetone induced a higher behavioural response than acetone alone for activation, release cage exit and directed flights, but the increases were not significant (Table 2). Acetone plus a mixture of 3-*n*-propyl phenol, 1-octen-3-ol and *p*-cresol at a ratio 1:4:8 dispensed from a polyethylene sachet did not improve the attraction of *G. brevipalpis* compared to acetone alone under the same wind tunnel conditions (Table 2).

Tsetse fly EAG responses to constituents of the neutral fraction of rumen fluid

The yield in the amine and neutral fraction of rumen fluid was low with our fractionation method as few compounds were detected by GC-FID (all products <10 µg from 15 ml of rumen fluid extracted). For these reasons, we were not able to satisfactorily characterize the constituents of this fraction. However, compounds such as terpenes and sulphides have already been regularly detected in rumen fluid (Clarke and Bauchop 1977) even though, compared to carboxylic acids, their quantity and quality is prone to vary depending on the ruminant's diet. Representatives of these chemical classes were tested here by recording EAG responses from tsetse fly antennae with known amounts of stimulants delivered from the GC column. Sulphides are also known to occur in animal intestines (Schöller et al. 1997) and both *G. fuscipes* and *G. pallidipes* responded to dimethyl trisulphide in a dose-dependent manner. By contrast, these two species showed no EAG response to dimethyl disulphide at any of the amounts tested (Fig. 4). Compounds from plants such as terpenes that pass untransformed through the digestive system can be found in rumen fluid and host breath. A mixture of terpenes comprising isocaryophyllene, *β*-farnesene and germacrene D elicited EAG responses in a dose-dependent manner from *G. pallidipes* in GC–EAD recordings (Fig. 5).

Discussion

The odour of bovine rumen fluid on its own was attractive to *G. pallidipes*, inducing a rapid response in the wind tunnel. The attractiveness of this substrate can be attributed to the range of volatile carboxylic acids, terpenes, phenols and indoles in the rumen fluid odour extract that induced EAG responses from *G. pallidipes*, *G. fuscipes* and *G. brevipalpis*. Moreover, as ruminants regularly void metabolites through eructation, we can suggest that these molecules are mixed with other host products such as acetone and CO_2 in host breath. This provides tsetse flies with a mixture of anaerobic and aerobic metabolites for host finding.

Indole, skatole and *p*-cresol were found persistently in our rumen fluid odour collections and elicited consistent EAG responses from *G. pallidipes*, *G. fuscipes* and *G. brevipalpis*. However, only *p*-cresol figured in our mildly acidic rumen fluid fraction. Previous studies have demonstrated phenolic compounds and indoles in the rumen (Clarke and Bauchop 1977) where their presence is attributed to the degradation by rumen bacteria of the amino acids tyrosine and tryptophan. Phenols, formed in

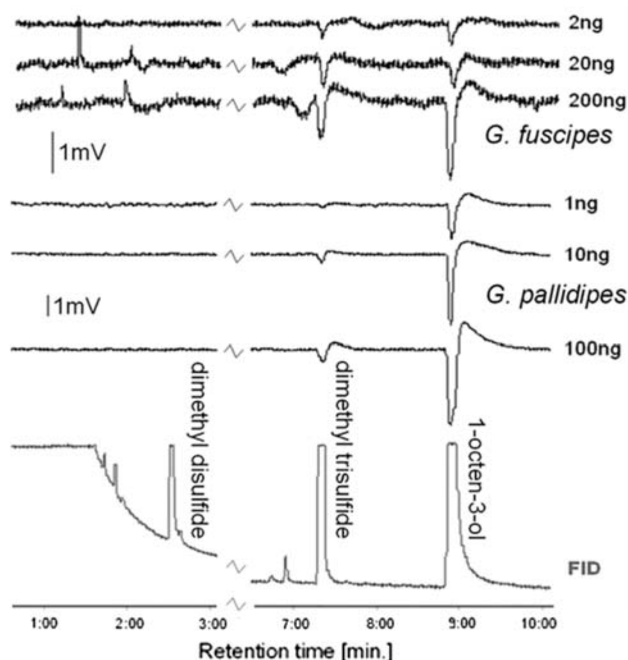

Fig. 4 Electrophysiological responses of *G. pallidipes* and *G. fuscipes* to different amounts of dimethyl disulphide, dimethyl trisulphide and 1-octen-3-ol eluting from a gas chromatographic column. The compounds delivered to the antennal preparations from a gas chromatographic column were tested at amounts of 1, 10 and 100 ng for *G. pallidipes* and at 2, 20 and 200 ng for *G. fuscipes*. 1-octen-3-ol was included for purposes of comparison. Note the absence of responses for dimethyl disulphide at any amount tested but the comparatively similar responses to dimethyl trisulphide and 1-octen-3-ol by the two species. The *scale bars* in mV provide a measure of the depolarisations recorded for each species. For more details see legend to Fig. 1

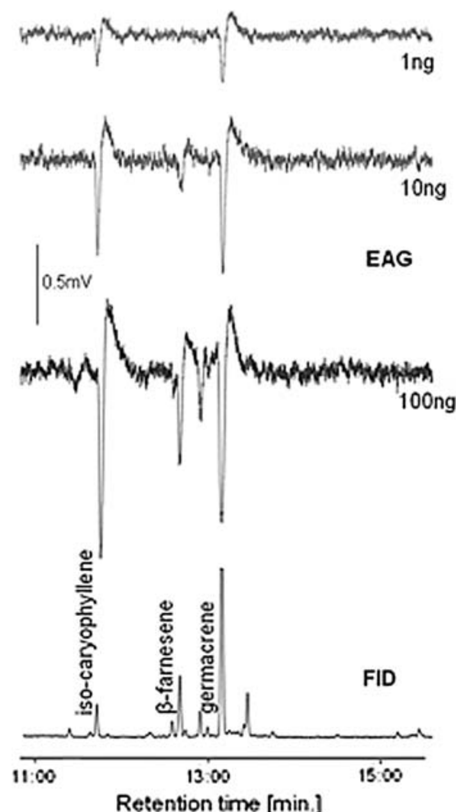

Fig. 5 Electroantennogram responses of *G. pallidipes* to a mixture of terpenes presented at different amounts (using germacrene D as reference) to the antennal preparations from a gas chromatographic column. The lower trace is the flame ionisation detector (*FID*) response of the chromatograph and the upper traces are the EAG responses (*EAG*) of the antenna olfactory cells generated during elution of the test mixture at different amounts from the gas chromatographic column. *Scale bar* in mV provides a measure of the depolarisations recorded

cattle urine due to microbial breakdown of glucuronates and sulphates (Okech and Hassanali 1990), were found to be the key compounds in urine to elicit attraction of morsitans and the palpalis group tsetse flies to this substrate (Bursell et al. 1988; Filledier and Mérot 1989). In particular, *p*-cresol and 3-*n*-propyl phenol were isolated from a bovine urine phenolic fraction and reproduced the attractive power of the mixture of phenols isolated from urine (Bursell et al. 1988). *p*-Cresol is currently used in combination to 3-*n*-propyl phenol, 1-octen-3-ol and acetone to bait visual traps and targets for tsetse flies (Vale et al. 1988; Filledier and Mérot 1989; Kappmeier and Nevill 1999) as well as for other haematophagous flies such as *S. calcitrans* (Cilek 1999).

Carboxylic acids are the major components of rumen fluid, and in our acidic fraction of rumen fluid 12 carboxylic acids were persistently recovered in reproducible amounts as seen in our collections from different seasons when cattle were fed on silage or fresh grass (data not shown). These acids are produced in large quantities in the rumen as end products of the degradation by microbes of plant carbohydrates and aliphatic amino acids, and are used as an energy source by ruminants (Clarke and Bauchop 1977; Mackie et al. 1998). The selective sensory responses of *G. pallidipes*, *G. fuscipes* and *G. brevipalpis* to these products is shown by the similarity in the EAG response amplitudes to similar amounts of the unbranched and branched C_2-C_7 carboxylic acids present in the acidic fraction of the rumen fluid and the absence of EAG responses to the aromatic carboxylic acids in this substrate. Carboxylic acids are not specific to the rumen bioreactor as they may also be released from protected areas of the host pelage where oxygen is limiting such as the axillary and genital areas. The common sensitivity to these compounds in the three different subgenera of tsetse flies tested here is probably linked to the survival value of sensing these acids in tsetse for tracking host animals. The mixture of butanoic, isovaleric, valeric and hexanoic acids at the ratio prevalent in rumen fluid (4:1:1:1), elicited significant levels of activation and cage exit, and some attraction for *G. pallidipes* in the wind tunnel compared to pure air. The proportions of these acids in mixtures tested seems to be important as the one that

mimicked the proportions present in rumen fluid increased the behavioural responses of *G. pallidipes* under the three behavioural criteria recorded more than an equimolar mixture of these acids. Tested singly with *G. brevipalpis* none of the carboxylic acids significantly activated the flies in our wind tunnel experiments, nor was there a significant change in any of the four behavioural criteria recorded when acetone was added. However, in these experiments acetone alone already induced significant levels of attraction in *G. brevipalpis*, and even adding the vapours of 3-*n*-propyl phenol, 1-octen-3-ol and *p*-cresol to acetone only lead to a slight but non-significant increase in the behavioural responses recorded. In the field the latter mixture permits to improve attraction of this species to visual target (Kappmeier and Nevill 1999). When carboxylic acids were added singly to field targets baited with either CO_2 (Vale 1980) or to a mixture containing 3-*n*-propyl phenol, 1-octen-3-ol, *p*-cresol and acetone in Epsilon traps (Torr et al. 1996) a slight decrease in attraction was recorded for morsitans flies. However, the amounts of these C_1–C_8 acids released in these experiments were 6–875 times higher than the amount of *p*-cresol released, and 1.4–250 times higher than the amounts of individual carboxylic acids released in our experiments. It is known that high concentrations of carboxylic acids can act as repellents for the malaria mosquito, *Anopheles gambiae*, where they can decrease the efficacy of approaches to mixtures that are otherwise active (Knols et al. 1997). Carboxylic acids and oxycarboxylic acids have already been reported as important chemostimuli in the sensory ecology of different haematophagous arthropods such as mosquitoes (Cork and Park 1996; Knols et al. 1997), sandflies (Dougherty et al. 1999), ticks (Donzé et al. 2004) and triatomine bugs (Guerenstein and Guerin 2001). It is quite possible that such acids may also influence tsetse fly behaviour, but the pertinent mixture and doses remain to be elucidated.

EAG responses to benzaldehyde were recorded for *G. pallidipes* and *G. brevipalpis* in the rumen fluid odour extract, and aldehydes are regularly found in breath of humans and cattle (Phillips et al. 1999; Spinhirne et al. 2004). Sulphides are known rumen fluid constituents and *G. fuscipes* and *G. pallidipes* showed EAG responses specifically to dimethyl trisulphides, whereas no EAG responses were recorded to dimethyl disulphide at any of the doses tested. As sulphides mainly arise from pathways involving the amino acids cysteine and methionine, they occur in small amount in livestock wastes (Mackie et al. 1998) and human emanations (Bernier et al. 2000). Sulphides have not yet been tested extensively on tsetse flies but sulphide sensitivity has been described in the tropical bont tick *Amblyomma variegatum* (Steullet and Guerin 1992), *Musca domestica* (Cossé and Baker 1996) and *S. calcitrans* (Jeanbourquin and Guerin 2007). Moreover, dimethyl trisulphide elicited increased landing by *Culex quinquefasciatus* and *Cx. nigripalpus* on a collagen casing treated with this compound (Allan et al. 2006) and attraction of *S. calcitrans* in a wind tunnel (Jeanbourquin and Guerin 2007), *Cx. tarsalis* to sticky traps (Du and Millar 1999) and seven species of *Calliphoridae* to traps baited with this substance (Nilssen et al. 1996).

The EAG responses of the three tsetse species from different African habitats to limonene, camphor, β-caryophyllene and β-selinene and by *G. pallidipes* to iso-caryophyllene and β-farnesene and germacrene D confirms that tsetse flies can sense terpene constituents of the rumen. Terpenes are released by plants upon foliage damage as happens during plant feeding and digestion by vertebrate hosts. As most of these compounds pass untransformed through the digestive system of ruminants, they occur in bovid rumen fluid and breath, but their identity in a particular host largely depends on the plant types the animal has fed on. Two terpenes that tsetse showed EAG responses to in this study, namely β-caryophyllene and germacrene D were previously identified in volatiles collected from the invasive plant *Lantana camara* (Syed and Guerin 2004) that is used by tsetse flies as a refuge from the high daytime temperatures of Africa (Okoth and Kapaata 1987). As terpenes from plants used as refugia by tsetse flies can also occur in hosts, these insects are probably making parsimonious use of chemostimuli to guide them to different resources.

The rumen of herbivores provides a rich profile of volatile compounds that are regularly eructed into the atmosphere and as such may provide cues for resource tracking by tsetse flies on ruminants. We have shown here that the peripheral olfactory systems of tsetse flies from three subgenera respond similarly to compounds that occur as predictable end products of rumen metabolism. Many of these chemostimulants merit further investigation as products affecting tsetse fly behaviour.

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