Vertebrate metabolites influence the sensory ecology of tsetse flies, *Glossina* spp.

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IMPRIMATUR POUR LA THESE

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Felix Kessler

« Le hasard ne profite qu'aux esprits préparés »

Louis Pasteur

«C'est en essayant encore et encore que le singe apprend à bondir »

proverbe africain

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Résumé

Mots clés : mouche tsétsé, *Glossina brevipalpis*, *G. pallidipes*, *G. fuscipes*, olfaction, électrophysiologie, comportement, tunnel de vol, vol en 3D, haleine, rumen

Les mouches tsétsé sont des diptères (Glossinidae) comportant 23 espèces divisées en 3 groupes. Ces mouches sont confinées en Afrique sub-saharienne et occupent trois biotopes préférentiellement : les forêts, la savanne et les abords des zones aquatiques.

Les deux sexes sont strictement hématophages et lors de leur repas sanguin les mouches tsétsé peuvent transmettre des trypanosomes responsables de la maladie du sommeil chez l'homme et de la Nagana chez les animaux.

Les mouches tsétsé localisent leurs hôtes grâce à des indices visuels tels que les contrastes, les formes et les couleurs à courte distance, et des indices olfactifs à distance. Les principaux composés olfactifs utilisés à l'heure actuelle sur le terrain sont l'acétone, l'octenol et deux phenols 3*n*-propyl phenol et *p*-cresol.

Comme les mouches tsétsé sont ovovivipares, la dynamique d'évolution de leur population est caractérisée par un lent renouvellement des générations. L'utilisation de pièges olfactifs et visuels peut permettre de décimer une population de mouche tsétsé en quelques années.

Comme les mouches volent très rapidement, nous avons filmé dans un tunnel de vol grâce à un système d'enregistrement 3D, les réponses de *G. brevipalpis* à l'haleine humaine en présence ou non d'une cible visuelle.

C'est en présence de cette cible visuelle placée dans la plume d'odeur que les vols de *G. brevipalpis* ont été les plus directs et lents, démontrant une forte interaction entre les systèmes de perception visuelle et olfactive de la mouche tsétsé.

Une majorité des herbivores sont des ruminants et constituent les hôtes préférés des mouches tsétsé. Les stimuli volatiles olfactifs issus du fluide de panse de bovins sont régulièrement éructés dans l'atmosphère et peuvent donc être utilisés comme indice pour les mouches tsétsé en quête d'un repas sanguin.

Les composés majoritaires du fluide de panse, les acides carboxyliques, sont détectés par les récepteurs olfactifs de différentes espèces de mouche tsétsé et modifient le comportement de vols de *G. pallidipes* et *G. brevipalpis* testés en tunnel de vol.

L'haleine émise par un hôte est, en elle-même, un attractant connu pour les arthropodes hématophages. L'attractivité de l'haleine humaine a été testée sur *G. brevipalpis* dans le tunnel de vol. Même s'il a été montré que le CO_2 joue un rôle major dans l'activation des mouches tsétsé, ce composé de l'haleine employé seul n'a pas engendré de comportement d'attraction dans notre tunnel de vol.

Cela suggère que d'autres composés présents dans l'haleine affectent le système nerveux olfactif des mouches tsétsé. Les réponses de ce système olfactif aux composés présents dans l'haleine humaine et de bovin tels que les aldéhydes, les terpènes, les cétones et des composés souffrés, ont été mesurés chez différentes espèces de mouches tsétsé par électroantennographie. Des réponses biologiques spécifiques des récepteurs olfactifs de trois espèces de mouches tsétsé ont été obtenues pour certains composés tel le decanal, le dimethyl trisulfide, et les stéréo-isomères du caryophyllene.

Les alkanes sont des métabolites présents dans l'haleine. Nous avons trouvé que *G. pallidipes* et *G. brevipalpis* sont capables de percevoir des alkanes de C_5 à C_{11} avec une sensibilité légèrement supérieure à celles de l'acétone qui est un chémostimulant connus pour ces insectes.

L'hexane ou l'heptane additionné aux composés utilisés sur le terrain : 1-octen-3-ol, 3*n*-propyl phenol et *p*-cresol ont montré un effet synergistique dans l'attraction de *G. pallidipes* et *G. brevipalpis*.

Summary

Key words : tsetse fly, *Glossina brevipalpis*, *G. pallidipes*, *G. fuscipes*, olfaction, electrophysiology, behaviour, wind tunnel, 3D tracking, breath, rumen

Tsetse flies are Diptera of the Glossinidae family with 23 species divided into three subgenera. They are currently confined in sub-Saharan Africa and occupy three preferred habitats: forest, savannah and riverine zones.

Both sexes are strictly haematophagous and during their blood meal they can transmit trypanosomiasis which causes sleeping sickness in humans and nagana in animals.

Tsetse flies locate hosts using visual cues including contrast, shape and colour at short range, and olfactory cues at distance. The major olfactory stimulants used nowadays in the field traps for tsetse flies are acetone, 1-octen-3-ol, 3*n*-propyl phenol and *p*-cresol.

As the tsetse flies are ovoviviparous, their population dynamic is characterised by a slow intrinsic rate of replacement, and so visual baited traps can decimate a tsetse fly population in a few years.

As tsetse flies are fast flyers, we filmed the responses of *G. brevipalpis* to human breath presented with and without a visual target in the wind tunnel using a 3D recording system.

In presence of a visual target placed in the plume of breath, flights of *G. brevipalpis* are the more directed and slower, demonstrating a high degree of interaction between tsetse fly visual and olfactory sensory inputs.

Herbivores are the preferred hosts for tsetse flies and among them ruminants occupy a dominant position. Volatile chemostimuli from the rumen fluid of cattle are regularly eructated into the atmosphere permitting these metabolites to be used as cues by tsetse flies searching for a blood meal.

The predominant compounds present, i.e. carboxylic acids are detected by olfactory receptor cells of tsetse fly species from different genera and they can serve to modify *G. pallidipes* and *G. brevipalpis* flight responses in the wind tunnel.

Breath emitted by a host is, on its own, a well known attractant for haematophagous arthropods, and the attractiveness of human breath was tested in the wind tunnel for *G. brevipalpis*. Even though CO_2 plays a major role as tsetse fly activator, this respiratory product failed to elicit significant behavioural responses on *G. brevipalpis* when presented alone, suggesting other products in breath affect the olfactory sensory system of tsetse flies.

Sensory responses of tsetse flies to products in different chemical families commonly found in human and cattle breath such as alyphatic aldehydes, terpens, ketones and sulphid compounds were recorded using the electroantennogram techniques. The EAG responses of three species of different subgenera responded in a similar manner, suggesting a specific sensitivity for compounds of these families such as decanal, dimethyl trisulfide and stereoisomer of caryophyllene.

Alkanes are volatile metabolites present in vertebrate breath. We found that *G. pallidipes* and *G. brevipalpis* are able to perceive C_5 to C_{11} alkanes with a sensitivity slightly higher than for acetone, a known tsetse fly chemostimulant. When hexane or heptane was mixed with the attractants 3*n*-propyl phenol, 1-octen-3-ol and *p*-cresol they had synergistic effect on *G. brevipalpis* attraction in the wind tunnel.

General introduction

Tsetse flies

Systematic & distribution

Tsetse flies are Diptera of the Glossinidae family. They are currently confined to 10 million km^2 in sub-Saharan Africa, but evidence of an earlier and wider distribution of tsetse and of their evolutionary (Williams *et al.*, 1992) age has been provided by the discovery of four fossil tsetse species from the Oligocene in Florissant shale in Colorado, N. America. Some authors have even suggested that tsetse may have had a role in the disappearance of some tertiary mammals of America. The present-day diversity of tsetse spp. with 23 species and 8 sub-species also testify to the role of habitat exploitation by vertebrates on the African-wide radiation in *Glossina*. Tsetse flies are divided into three subgenera, based on genetic and allozyme variation (Gooding *et al.*, 1991), which correspond to three preferred habitats (Jordan, 1986). Members of the subgenera Glossina (*morsitans* flies with 5 spp.) occupy woodland savannah areas, their wild hosts are bovids and wild pigs and this group includes the more important vectors of animal trypanosomiasis such as *G. pallidipes*, *G. morsitans* subspp., *G. longipennis* and *G. austeni*. Species of the subgenera Nemorhina (*palpalis* flies with 5 spp.) occur in lowland rain forest, along riverbanks and lakeshores in close ecological association with reptiles as well as with wild ruminants and birds and they includes important vectors of human sleeping sickness such as *G. fuscipes* and *G. palpalis* subspp.. Members of the subgenera Austenina (*fusca* flies with 13 spp.) inhabit lowland rainforest and gallery forests and because their natural habitat is less used by livestock and people their typical hosts are forest pigs, and they are less implicated as significant vectors of animal trypanosomiasis even if some species such as *G. brevipalpis* cause problems in animal health (Fig. 1).

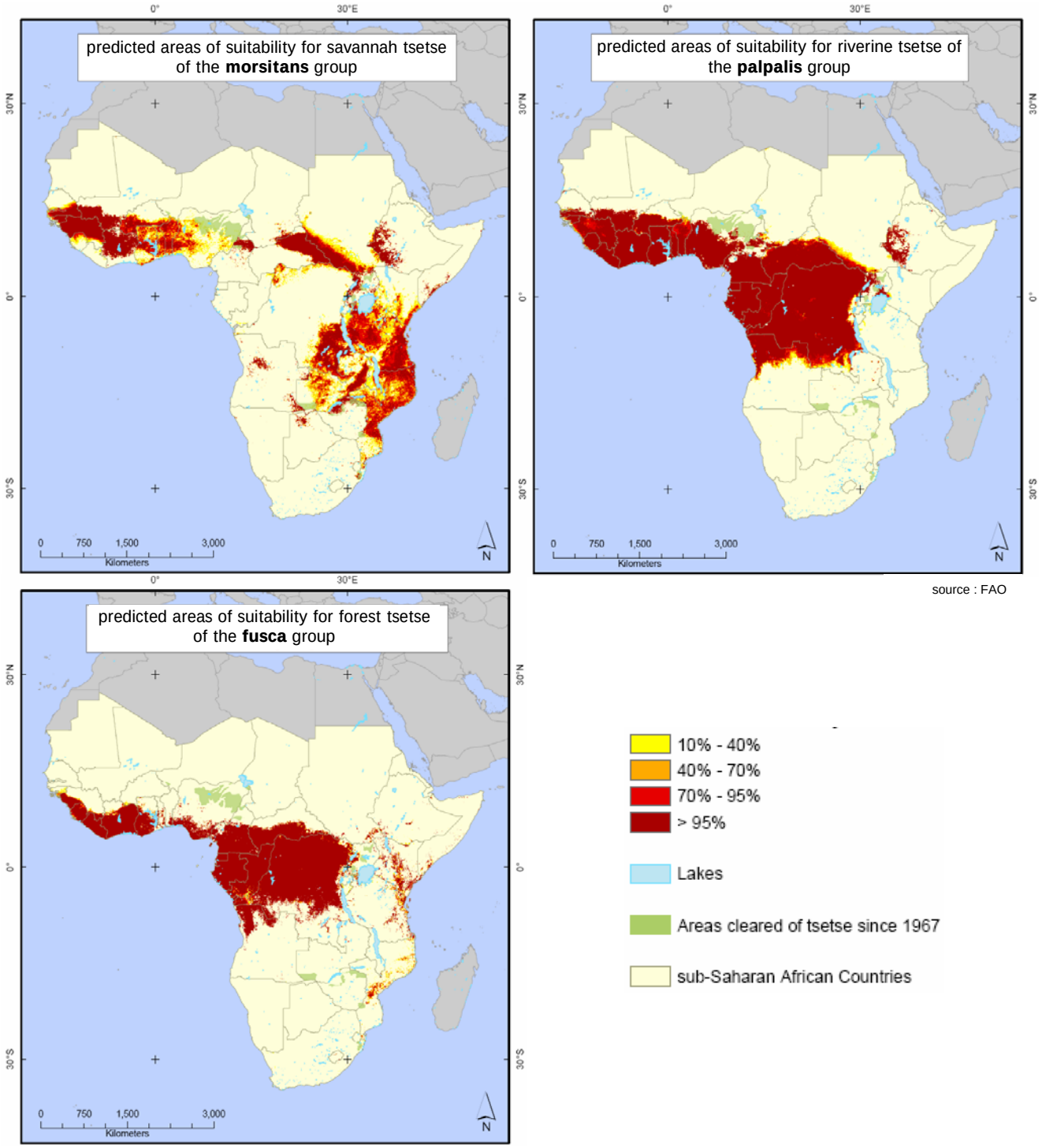

Figure 1. Predicted areas of suitability for tsetse flies of the 3 subgenera.

Maps produced for FAO Animal Health Programme by the Environmental Research Group Oxford in collaboration with the Trypanosomosis and Land Use in Africa research group at the Department of Zoology, University of Oxford in November 1999. The modelling process relies on logistic regression of fly presence against a wide range of predictors. The predictor variables include remotely sensed (satellite image) surrogates of climate such as vegetation, temperature and moisture. Demographic, topographic and agroecological predictors are also used. The prediction was created at a 5 kilometers resolution for the whole sub-Saharan Africa.

Description of the flies

Tsetse flies are medium to large biting flies (5-20mm) distinguishable from other Diptera by four characteristics of their anatomy. The most visible are the complete folding of their wings above the abdomen when at rest and the distinctly long proboscis starting at the thecal bulb underneath the head. Two other less apparent characteristics are the branched hairs of the arista and the hatchet shape in the central cell of the wings.

Tsetse flies are diurnal, with both sexes hazardously seeking a blood meal at regular 2-3 days interval. Their total daily flight time is short, and, apart from searching for a host tsetse flies must seek cover from high daytime temperatures in Africa. The pattern of their activity is linked to an endogenous rhythm of spontaneous activity (Brady, 1972) shared between species, with activity peaks during morning and evening (Crump & Brady, 1979 and Annex X) with some exceptions.

The blood meal provides all the energy, protein and water needed for maintenance and reproduction (Annex C). The female fly feeds a developing larva in its abdomen via milk glands and can give birth every two weeks or so to a mature larva that burrows into the soil to pupate. A female produces no more than 10 offspring in her lifetime. This high investment in young by female tsetse flies gives rise to a slow increase in population growth.

Host

Tsetse flies locate hosts beyond their visual range by following olfactory cues. Their olfactory perception extends to 60-120m downwind (Vale, 1977) and visual cues including contrast, shape and colour are important in host location at a shorter range of 10m (Gibson & Torr, 1999; Vale, 1974). Tsetse flies are able to feed on various hosts, but seem to acquire a feeding preference influenced by the host selected for their 1st meal (Bouyer *et al.*, 2007). As for many other haematophagous insects, herbivores are among the preferred choice, due to their habits of herding and their sessile nature. The terrestrial ungulates, especially ruminants, occupy a dominant position as eutherian herbivores whose eco- and morpho-physiological diversification permits them to feed on all types of plants. Their chambered stomach contains a variety of microorganisms that hydrolyze plant polymers and ferment monomers to yield energy, permitting these mamals to exploit wide ranging areas of vegetation (Fig. 2). The high diversity of compounds produced in this gut bio-reactor by microbes is not specific to ruminants and can be released from protected areas of the body of other host animals where oxygen may be limiting such as the axillary and genital areas.

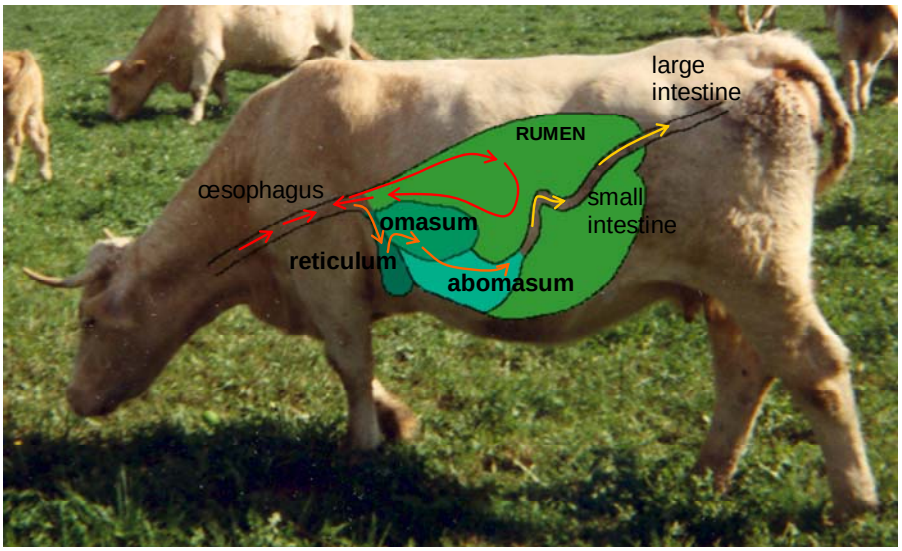

Figure 2. An example of chambered stomach of the cow

The rumen, the first chamber of the forestomach, occupies a large part of animals viscera.

Problems caused by tsetse fly

The tsetse fly and the diseases it transmits, African trypanosomiasis, cause a problem for human and agricultural systems, that is unique in the world: African trypynosomiasis affects both man and his livestock. It is protozoans of the genus *Trypanosoma* which cause these diseases. The fly becomes infected when it feeds on an infected host and the disease is transmitted via the salivary glands when infected flies feed on a new host. Nagana occurs in livestock throughout the tsetse fly belt, whereas sleeping sickness in humans is restricted to a number of local foci (Fig. 3).

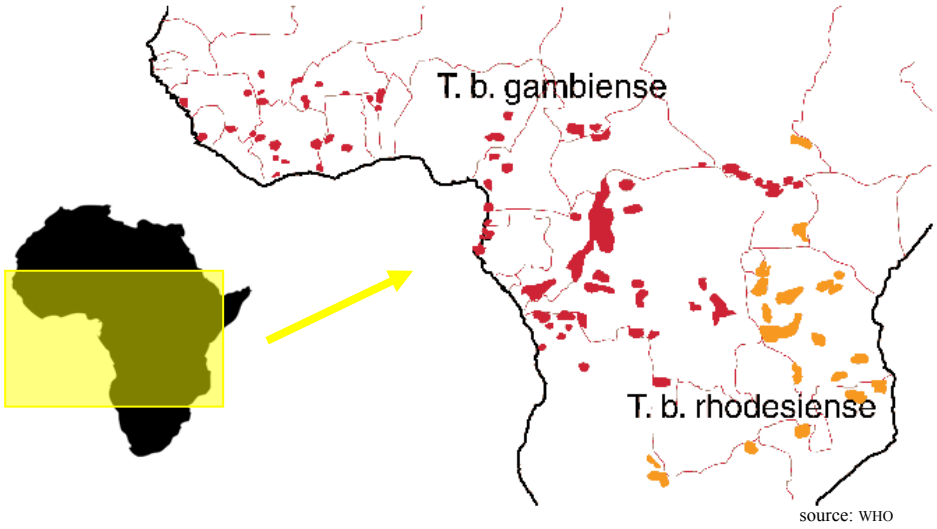

Figure 3. Map of the principal local foci of human sleeping sickness in Africa and dominant trypanosome species responsible for the disease.

Due to nagana, the maintenance of productive cattle in the presence of tsetse is very difficult, in some areas even impossible. Nagana not only affects supplies of meat and milk but also limits the development of livestock farming in Africa. About one third of the 150 million cattle and tens of millions of goats and sheep are at risk of becoming infected with the disease, and 2 to 3 millions heads of cattle die of the disease every year. The World Health Organisation estimates that 55-60 million people are at risk of human trypanosomiasis, with between 50'000-70'000 infected and 20'000 deaths in 2005. The alarming observation is that tsetse and trypanosomiasis is a more serious problem today than some 50 years ago when the first generation of insecticides was in use (Fig. 4).

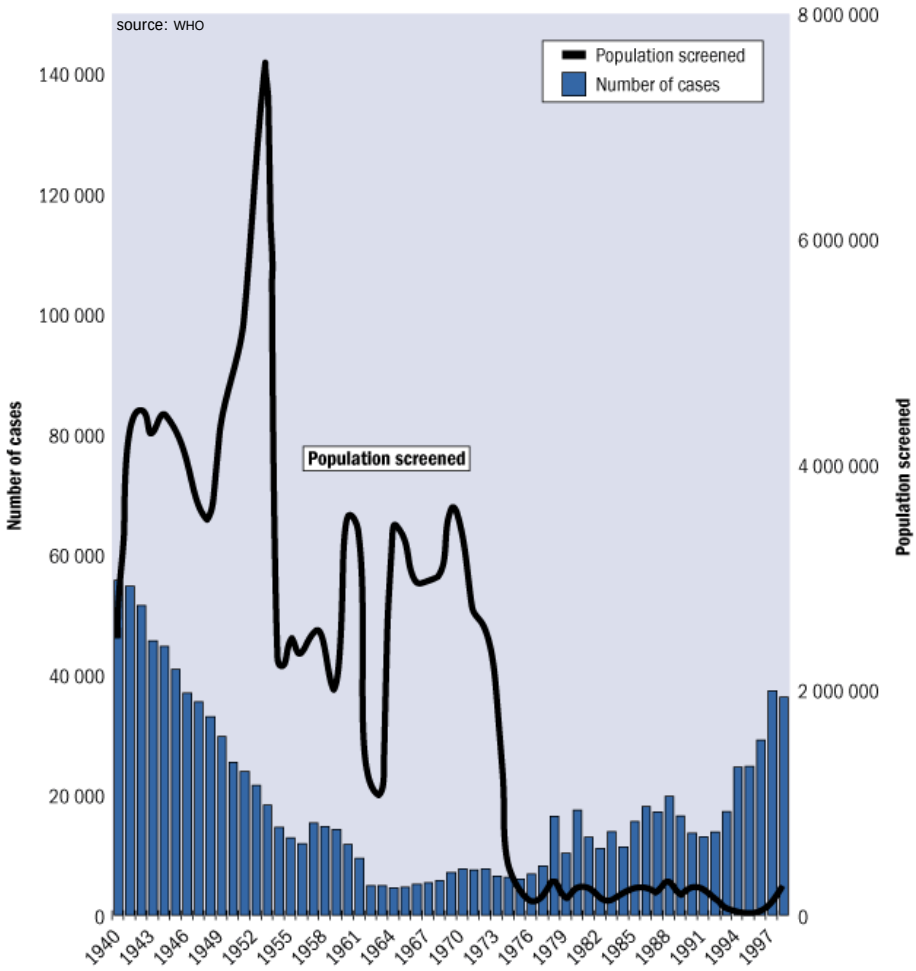

Figure 4. Number of reported cases of human African trypanosomiasis and population levels screened between 1940-1998

Tsetse flies control

Tsetse control up to the middle of the 20th century relied on bush clearing, selective wild game animal destruction and spraying with insecticides. Although trapping devices to control tsetse flies have been known since the early part of the last century, they did not come into common use until the second half of the 20th century when ecological considerations regarding the use of insecticides and the destruction of tsetse habitat and game animals became an issue. A wide range of trap designs has been developed for tsetse flies (Vale, 1974), all based on the perceived shape and colour discrimination of tsetse flies, and on the contrast between trap and background vegetation: combinations of blue and black cloth give the best results (reviewed by Green, 1994). A wide variety of traps exists, with different designs being more attractive to some species: the NGU, the epsilon and the NZI traps for savannah species (Brightwell *et al.*, 1987; Mihok, 2002), the biconical and the pyramidal traps for riverine species (Challier & Laveissiere, 1973), the H-trap, the biconical and the epsilon traps for the fusca species (Kappmeier, 2000). Simpler than traps, insecticide-impregnated screens named targets can be used to reduce tsetse fly population but do not permit entomological surveillance and control. Moreover, traps and targets can provide a locally produced tool for practical and environmentally acceptable tsetse control in rural Africa.

Traps alone have played a significant role in tsetse control and monitoring, but a significant advance came with the development of odour baits that can significantly increase the attractiveness of these devices. Indeed, the efficiency of the system depends on the numbers of targets and traps deployed relative to the abundance and particular foraging and dispersal habits of tsetse. Dransfield (1984) indicated a limit in the range of attraction for unbaited stationary traps at less than 20m, and Williams *et al.* (1992) found that the probable limit of attractiveness for most odour-baited trap to be 100m. The addition of odour permits an improvement in trap efficiency and consequently, deployment requirements by decreasing the trap density (and so costs) without reducing the likelihood of fly capture.

Research on the sensory ecology of tsetse flies has concentrated on one or a few products produced by host animals: acetone from breath (Bursell, 1984a), 1-octen-3-ol from the whole body (Hall *et al.*, 1984) and 3*n*-propyl phenol and *p*-cresol isolated from urine (Owaga, 1985; Bursell *et al.*, 1988; Vale *et al.*, 1988). In the field, these semiochemicals are placed near the visual trap, with 3*n*-propylphenol, 1-octen-3-ol and *p*-cresol at a ratio 1:4:8 dispensed from a polyethylene sachet (with respective release rates of 0.5mg/h, 0.2mg/h, 1.5mg/h) plus acetone evaporating from an open glass bottle (with release rate of 150-2500mg/h ; FAO, 1992).

Moreover, the benefits of baiting traps with odour attractants vary between species, mainly because research to date has concentrated on testing chemostimuli for a few savannah species, i.e. *G. morsitans morsitans* and *G. pallidipes*. Mixtures of the compounds mentioned in *table 1* significantly augment catches of tsetse flies of the *morsitans* group, but these attractant combinations do not significantly increase catches of *fusca* and *palpalis* spp. (Mwangelwa *et al.*, 1991; FAO, 1992; Green, 1994) even though some species in these tsetse fly groups respond well to some mixtures.

Table 1. Host odour compounds and their combinations currently used to improve trap catches of tsetse fly spp. from different subgenera.

		visual trap +A		visual trap +A +O		visual trap +P +O +C		visual trap +P +O +C +A	
morsitans	*G. pallidipes*	2 - 6	adg	3 - 5	ad	1.1 - 1.8	c	4 - 20	eg
	G. morsitans	2 - 6	a	2 - 3	ab			3 - 10	eg
palpalis	*G. tachinoides*	1 – 1.2	bf	0.3 - 1.2	bfg			0.9 – 2.3	g
	G. palpalis			0.5 – 1.2	g			0.6 – 1.2	g
fusca	*G. longipennis*	2	d	1.6 - 5	def	0.9 - 1.4	e		
	G. brevipalpis	0.5	g	0.2	g			0.7 – 1.8	g

A=acetone, **O**=1-octen-3-ol, **C**=*p*-cresol, **P**=3*n*-propylphenol
ref.: **a**-VALE & HALL 1985, **b**-MEROT *et al.* 1988, **c**-BRIGHTWELL *et al.* 1991, **d**-BAYLIS & NAMBIRO 1993, **e**-GREEN 1994, **f**-SPÄTH 1995, **g**-IAEA 2003.
The numbers are catch indices defined as the daily mean captures in traps with odour baits relative to control traps without odour.

An other control method used is to directly apply insecticides to cattle. Even if this does not prevent tsetse flies from biting, flies in contact with treated animals die and this technique permits to kill about as many tsetse flies as a single odour-baited target. However, because insecticide-treated cattle occur in herds, only a confined areas of control is covered (Hargrove *et al.*, 2000). At low population densities the sterile insect technique is more effective than the traditional methods cited above. It consists in rendering reared tsetse fly males sterile by gamma irradiations and releasing them in the field in large numbers where they successfully mate and compete with wild tsetse flies. However, because a ratio of more than 100 sterile males to one wild male is needed for success (Leak, 1999) and re-invasion of the treated zone can not be excluded, an integrated approach with different insect control systems operating at a regional level is needed to establish zones for sustainable agriculture practice. Today, baited-traps and targets are the only ecologically viable and efficient method to establish barrier systems to prevent the re-infestation of tsetse free zones (Politzar & Cuisance, 1983; Muzari, 1999).

Sensory ecology

The "ecology" section of discipline treats the relationship of living organism to their environment whereas the "sensory" section refers to functions of sense organs and to their influence on behaviour in the same environment. The integration of the two focuses on how and why useful information are obtained and exploited by an organism from the environment.

Senses in arthropods

As it was shown above with tsetse flies, insects use a number of sensory cues in habitat and host location and selection including visual, olfactory, gustatory and mechanical stimuli, as well as temperature, humidity and light intensity. Haematophagous arthropods provide a good example of how these various senses are employed. Hunter ticks such as *Hyalomma dromedarii* equipped with a rudimentary visual system consisting of a lens fixed over a small field of photoreceptors can home in on certain silhouettes (Kaltenrieder, 1990), and it is known that flies such as *Tabanidae* spp. or *Glossinidae* spp. have a highly developed visual system (Hardie *et al.*, 1989). Heat radiation from the host can provide an orientation cue to ticks which possess thermoreceptors on the tarsi of the first leg pair that they wave in the air during active search behaviour (Hess & Loftus, 1984), and night-active triatomine bugs which can discriminate between objects at different temperatures (Lazzari & Núñez, 1989). It has been established for over half a century that male *Culicidae* spp. in search of a mate employ the tone of the female wing-beat (Roth, 1948) and tsetse flies are able to produce sounds (Annex D). But among the sensory adaptation of insects, chemosensory systems play a leading role.

Olfactory receptors cells in chemosensilla on appendages such as the antennae of insects and the first leg-pair of ticks permit the detection of volatile molecules from a distance that can modify behaviours. Several products have been implicated in the host relations of these organisms. These include oxidation products such as CO_2 from vertebrate breath for mosquitoes, ticks and tsetse flies (Braverman *et al.*, 1991; Steullet & Guerin, 1992b; Evans & Gooding, 2002), 1-octen-3-ol from host odour for tsetse flies (Hall *et al.*, 1984) and fermentation products such as 3*n*-propylphenol and *p*-cresol from urine for tsetse flies (Saini & Hassanali, 1994). Other compounds such as ammonia were also identified as host attractants for triatomine bugs and mosquitoes (Taneja & Guerin, 1997; Geier *et al.*, 1999). Mosquitoes must regularly seek plants as they depend on nectar as a source of energy for flight and Bowen (1992) found that *Culex pipiens* females can detect terpenes with antennal receptor cells, and behavioural assays suggested a possible role for these compounds in nectar feeding by this species. In the quest for new attractants, researchers have also investigated products present in microbial cultures (Knols & De Jong, 1996; Meijerink *et al.*, 2000). Particular attention is given to aliphatic acids. The unusual carboxylic acids on human skin have long been recognised as fermentation products that

might contribute to specificity for anthropophilic mosquitoes (Nicolaides, 1974). Cork & Park (1996) demonstrated that 22 carboxylic acids in human sweat fractions activated olfactory receptor cells on the antenna of *Anopheles gambiae*, the major vector of malaria in Africa, and a mixture of these acids also attracts this mosquito species (Knols *et al.*, 1997). In addition, mixtures of the above mentioned compound classes prove important: traps baited with 1-octenol-3-ol have no significant effect on the catches of tsetse flies, but when traps are baited with 1-octenol-3-ol and other host stimuli, catches are significantly increased (Vale & Hall, 1985). Geier *et al.* (1999) showed synergism in the attraction of *Anopheles* mosquitoes spp. to CO_2 plus lactic acid. Similar effects occur between CO_2 and the aggregation-attachment pheromone made up of ortho-nitrophenol, nonanoic acid and methyl salicylate that is employed by the tick *Amblyomma variegatum* to attract conspecifics to a suitable host (Barré *et al.*, 1997). Further insight into how components of host odour interact was provided by Geier *et al.* (1999) and Bosch *et al.* (2000) who demonstrated the absolute requirement of lactic acid in the orientation of the yellow fever mosquito, *Aedes aegypti*, in a mixture of NH_3 combined with carboxylic acids.

What is emerging from research in this area is that host recognition from a distance by haematophagous arthropods is based on sensitivity to combinations of products of different functionality arising from various metabolic pathways of their hosts.

Physiology of olfaction in arthropods

As the neural organisation of insect olfaction ressembles that of vertebrates, as the number of cells of the neuronal network is reduced and as the genome of some insect is now known, insects provide an attractive system to study olfaction from the molecular to the behavioural level. Studies on *Drosophila* fruit flies permit genetic identification of candidate odorant receptors. Moreover, recent reviews have highlighted the importance of research into the olfactory responses of bloodsucking insects to host odours for a better understanding of vector behaviour and for identifying more effective trapping methods for monitoring or control purposes.

In arthropods, the olfactory receptor cells are bipolar neurons with dendrites ramifying in a cuticular apparatus called a sensillum. The number of neurons in olfactory sensilla varies among the different types of sensillae and between insect species. Sensillae are found all over the arthropod body, but the olfactory sensillae are more concentrated on specialised body appendages such as palps and antennae of insects, or the first leg-pair of ticks. These sensillae are of different types and are perforated by numerous small pores through which odor molecules pass through the cuticule and reach underlying sensory neurons. The receptor lymph, isolated from the haemolymph, surrounds olfactory receptor cells in sensillae and in most cases olfactory

processing starts with odour-binding proteins transporting the odour ligands to their olfactory receptor (Vogt & Riddiford, 1981). These odorant-biding proteins may act as a first filter permitting odour discrimination (Leal, 2003). When the odour molecule binds to the receptor site of the olfactory receptor, a G-protein with seven transmembrane domains induces a cascade of reactions is initiated. Second messengers are liberated and activate the opening of cation channels, generating a depolarisation called the receptor potential. This electrical signal travels along the dendrite and reaches the impulse generator site in the soma of the receptor cell, where it initiates action potentials that propagate to the brain via the axon. Olfactory neurones responding to the same ligand project to the ipsilateral antennal lobe where they form synapses with projection neurons and local interneurons, forming a glomerulus.

Sensory physiology

The electroantennogram (EAG) introduced by Schneider (1957b) permits the measurement of the electrophysiological response of an insect antenna to stimulation with an odour. In our case, EAG potentials are recorded with glass capillary electrodes, between the reference grounded electrode inserted inside the pedicel through the cut head and the recording electrode placed on the tip of the funiculus with micro manipulators (Fig. 5). The recording electrode is connected to

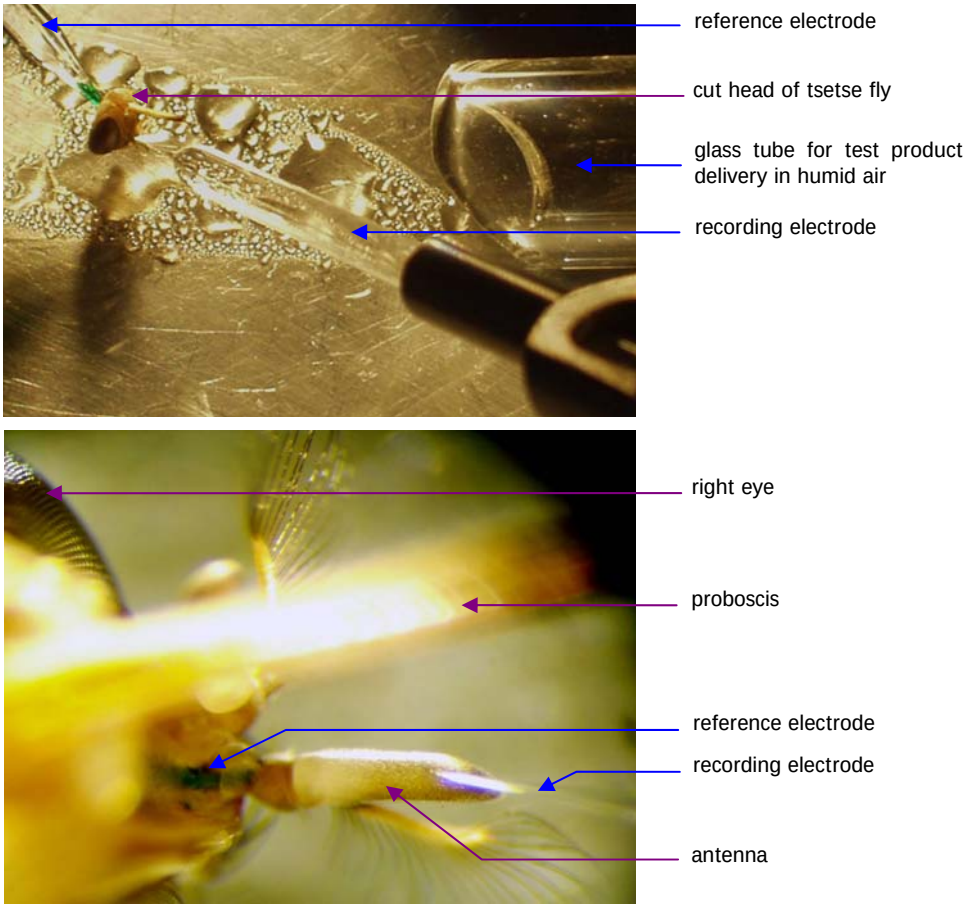

Figure 5. Photos with detail of a *G. brevipalpis* cut head during electroantennogram recording with glass capillary electrodes

The reference electrode is inserted inside the pedicel through the cut head (left) and the recording glass capillary electrode is placed on the tip of the funiculus (right).

a high impedance preamplifier to record the electrophysiological potential generated when the stimulus is injected into a humid air stream flowing other the antenna. Because tsetse fly EAG responses have been shown to correlate well with fly behaviour (Hall *et al.* 1984, DenOtter *et al.* 1988), the EAG is a very useful laboratory method to investigate the effects of candidate chemostimuli on tsetse spp. and to compare tsetse fly sensitivities to different products and their doses.

Above all, coupling the antenna as a biological detector (EAD) to the effluent of a high resolution gas chromatographic column (GC) permits the localization of active components from relevant biological substrates with a very sensitive and selective detection system (GC-EAD ; Moorhouse *et al.*, 1969). Here the insect antenna is used as an on-line detector in parallel with the flame ionisation detector of the gas chromatograph, with half of the amount injected sent on the antennae. The high sensitivity of the insect antenna can permit analysis for trace components not detectable by physicochemical means. The short elution times of the constituents of extracts from the capillary column insures that there are no adaptation effects on the part of the antennal olfactory receptor cells. Moreover, the same gas-chromatographic column can then be coupled with a mass spectrometer (GC-MS) to establish the chemical identity of biologically active components of the extract.

Behaviour

The wind tunnel provides a near physical mimic of the environment where factors such as temperature, humidity, light, wind velocity can be manipulated to produce a particular experimental situation. Test stimuli are delivered at the upwind end of the wind tunnel and carried by the laminar air flow downwind to where the insects are released (Fig. 6). If the

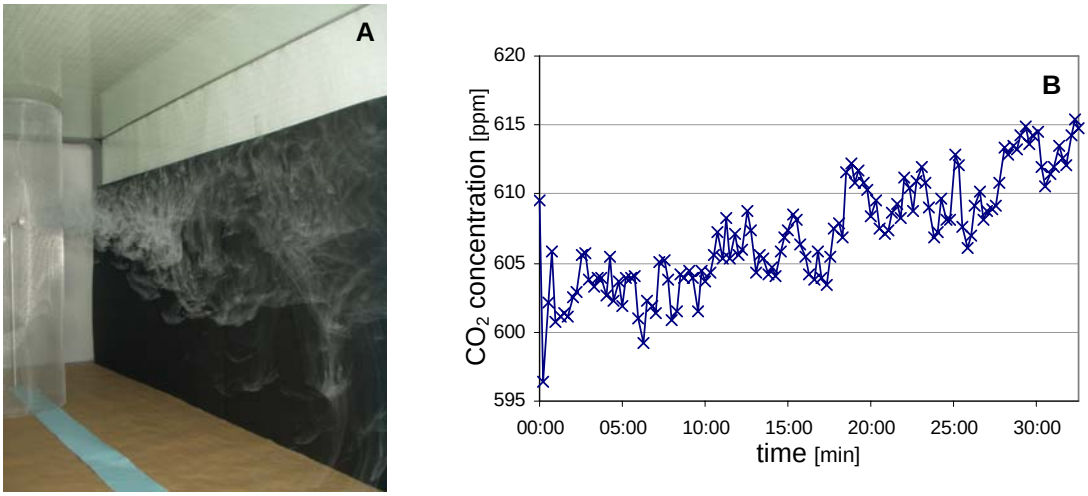

Figure 6. Visualisation of a plume of odour in the wind tunnel used for these studies

The plume of ammonium acetate (A) was made by mixing acetic acid and ammonia. Note how the plume is formed of discrete bursts of ammonium salt. The CO_2 recording at the downwind end of the wind tunnel (B) shows considerable variations which can be linked to the plume structure. The overall increase in CO_2 level is due to the injection of CO_2 into the wind tunnel.

stimulus activates the insect, it subsequently progresses upwind in the plume of chemical to the source. To quantify the behavioural responses of an insect, visible events such as activation, exit from the release cage, directed flight in the plume of odour and attraction to the source can be quantified. In addition, the use of cameras permits to quantify parameters such as velocity, distance travelled, acceleration, straightness, angular sum and angular velocity of the insect. In this manner the flight responses triggered by different odours can be more precisely qualified.

Diffusion of an odour in an air stream forms a plume which consists of a serie of bursts of odour that varies in concentration, duration and rate of repetition (Murlis & Jones, 1981). The structure of the plume does not change much with distance, even though the wind and the environment may modify its form. In our laboratory wind tunnel, the plume can be visualised using ammonium acetate (Fig. 6A). Recording the plume created by releasing CO_2 from a gas tank containing 2% of CO_2 in air at the upwind end of the tunnel (Fig. 6B) provided results similar to those of Zolner *et al.* (2004) in the field.

Humans have used this ability of insect to detect volatile molecules from a distance to develop insect behaviour-controlling chemicals with the aim to attract or repel arthropods. One of the most important applications of attractants is in crop pest management where pheromones which attract males to a synthetic blend of female sex pheromone components are used for pest monitoring purposes and also for the control of crop pests using the sex pheromone based confusion technique. Another application of behaviour-controlling chemicals for insects is to increase the efficiency of visual trapping systems for winged blood-sucking insects like tsetse flies as it was explain above.

Hypothesis of the thesis

It is well known that olfaction plays a major role in blood meal detection by tsetse flies from a distance, whereas vision prevails at short range. We assumed that the flight behaviours performed by tsetse flies progressing towards a host depend on the olfactory and visual sensory canals. To test this, a 3D tracking system recording a range of flights parameters was used to analyse tsetse fly behaviour to human breath in the presence and absence of a visual target (Chapter 1).

As presently used odours permit attraction of only some tsetse fly species to visual devices in the field we aimed our research to the identification of new host metabolites that tsetse flies species may be able to perceive and that may modify their flying behaviour.

Tsetse flies are attracted by compounds of plant origin and herbivores constitute the major group of animals providing blood meals for tsetse flies. We hypothesised that tsetse flies could make parsimonious use of plant secondary products. To test this hypothesis we researched the rich content of the first stomach chamber of cattle which contains a large variety of untransformed plant compounds as well as anaerobic products formed during the degradation of plant tissues (Chapter 2).

Furthermore, as volatiles from the digestive system are regurlarly eructated into the atmosphere via breath and since breath itself, containing both anaerobic and aerobic products, is a known attractant for haematophagous arthropods, we decided to test this readily available substrate to isolate other products that tsetse flies may be able to detect (Chapter 3). Alkanes, in particular, constitute a family of metabolites present in breath whose attractiveness to insects has not been investigated in detail. (Chapter 4).

The aim of this thesis is to improve our knowledge of odour perception in an important insect vector of disease. The practical outcome would be to identify products that could prove useful to develop attractive devices that require a minimum of maintenance in the African field setting.

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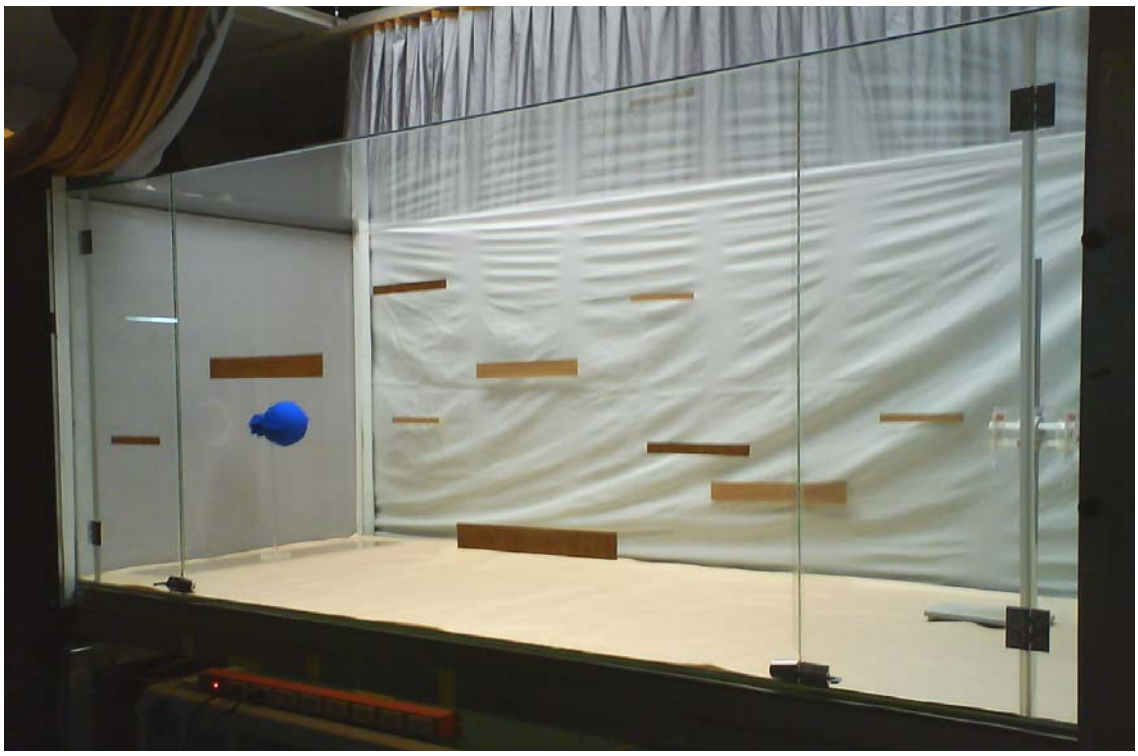
Chapter 1



3D tracking of *Glossina brevipalpis* in a wind tunnel as a tool to study the flight behaviour of tsetse flies stimulated by different parameters.

Abstract

As tsetse flies are fast flyers, video recording have already been carried out on tsetse fly flight responses both in the field and in wind tunnels. The aim of such research is to study the way tsetse flies locate and approach hosts and visual targets. Such knowledge can help in the development of better trapping devices. Using a 3D video recording system we filmed the responses of *G. brevipalpis* to human breath presented with and without a visual target in a wind tunnel in order to undertake quantitative analysis of the flight responses of tsetse flies to treatments.

The most notable aspect of the flights recorded for *G. brevipalpis* are the more directed and slower flights in the presence of a visual target placed in a plume of human breath borne in the wind. The turning rate during such directed flights is probably influenced by breath components the predominating role of the visual component was confirmed by the manner in which *G. brevipalpis* regulates flight within stricter limits in the presence of a visual target. The 3D recordings show that tsetse flies are capable of complex behaviours during host searching, with a high degree of interaction between the visual and olfactory sensory inputs governing their overall flight responses.

Key words : tsetse flies, wind-tunnel experiments, tracking 3D, olfaction, vision.

Introduction

Numerous studies have already been carried out on tsetse fly flight responses both in the field and in wind tunnels. The different modes of host detection by tsetse flies are influenced primarily by habitat and wind speed (Williams, 1994). In an open habitat with relatively strong and steady winds tsetse flies are able to use wind as a cue to assess the direction of the odour source (anemotaxis) because the odour information forms a coherent and directional plume with 80% of the odour arriving within an angle of 10° at a distance of 15m from the source (Brady *et al.*, 1989). Gibson *et al.* (1991) observed that in the field the flights of *Glossina m. morsitans* and *G. pallidipes* were strongly biased in the upwind direction in the presence of an odour and downwind with the absence of one. On the other hand, when the wind is very variable or the vegetation is dense, no useful directional information is carried by the wind. Brady *et al.* (1989) recorded a negative correlation between wind speed and air direction and measured 4 times more wind meander between open woodland with shrubs and open ground without cover. Only 30% of the odour released carried information regarding the true source direction at 15m downwind. It was observed both in field and laboratory studies that in such a case tsetse flies did not undertake anemotaxis but change their flight behaviours depending on whether they are or are not in the presence of an odour by klinokinesis, namely increasing the turning rate to locate an odour source (Gibson & Brady, 1988; Brady *et al.*, 1990; Williams, 1994). Crosswind flights permit acquisition of a maximum amount of information, but Sabelis & Schippers (1984) have suggested in a model treating within-odour plume searching strategy linked to energy cost that if the wind fluctuates by more than 30° from its mean direction then upwind or downwind searches as observed by Gibson *et al.* (1991) for *G. m. morsitans* and *G. pallidipes* are the best strategy.

The main observations in the field are that when tsetse flies loose contact with an odour plume they turn sharply around (Bursell, 1984a; Gibson & Brady, 1985; Torr, 1988a), doing so by flying left and right of due upwind when the odour concentration indicates that the host is only a few meters away (Gibson *et al.*, 1991). The interpretation of this flight strategy is that it permits the fly to get within visual range of a host (Gibson *et al.*, 1991), for in the final phases of approach to a visual target tsetse flies are guided by visual cues (Bursell, 1984b). This was demonstrated by Torr (1988a; 1989) who showed that in the absence of visual cues tsetse flies always tend to overshoot the odour source, and flies flying in an odour plume towards a visual target will not turn around should they lose contact with odour but head on for the target. As Torr (1988b) and Williams (1994) explained, the way in which flies locate and approach traps is important, as such knowledge could aid in the development of bioassays for olfactory stimulants. Nevertheless, it is impossible to specify the precise location of an odour plume at a given time in the field due to the vagaries of the wind, and this is why some researchers decided to make their

investigations under wind tunnel conditions where parameters can be precisely controlled. For example, Evans & Gooding (2002) studied the threshold of temperature, humidity and CO_2 fluctuations that generated anemotactic flights response of *G. m. morsitans* and, above all, concluded that an increase of thus 0.0051% of CO_2 above background is sufficient to significantly increase the upwind flight response of this insect.

Because our behavioural tests had to take place during the 2 hours of highest activity in the day (Annex B) and were all made with one insect at a time to avoid disturbance between flies (Warnes, 1989), only a few flies could be tested in any given session. As tsetse flies are very fast flyers, it was difficult for the human eye to record quantitative behavioural differences of flies flying to different treatments. To the human observer only occurrence of specific behaviour criteria can be employed to characterise tsetse behaviour such as *activation* (the fly moved in the release cage), *exit* (the fly flew from the cage), *directed flight* (the fly undertook flight of at least 50cm upwind flight in the odour plume) and *attraction* (the fly made a directed flight to within 10cm of the source). Such recordings give rise to binary data sets, i.e. *yes* or *no* data categories for each of the behavioural criteria, a factor that limits the choice of statistical tests that can be applied. Even when high numbers of flies are tested for a given treatment, variations in the behavioural responses observed between days remain high (Tab. 1), rendering it difficult to find significant differences between treatments (Annex H). We calculated that to observe a significant difference between two substances attracting 50% and 30% of flies respectively, at least 180 flies would have to be tested for each substance.

Table 1. Mean behavioural responses and corresponding standard deviation values over days for the responses of *G. brevipalpis* and *G. pallidipes* to different treatments

			activation [%]		exit [%]		attraction [%]		number of days	mean flies/day
			MEAN	SD	MEAN	SD	MEAN	SD		
G. brevipalpis	glass U tube	DCM	18.5	13.5	10.1	9.7	3.4	5.6	24	13.1
	filter paper in 1L gas-wash bottle	POCA [1]	26.5	19.4	21.1	18.0	7.5	13.0	8	10.1
		POCA [2]	28.9	16.4	18.5	15.6	11.3	13.2	8	11.1
G. pallidipes		POC	21.7	14.1	16.7	7.5	16.7	7.5	4	8.3

For all tests charcoal filtered air (0.5L/min) was used, passing through a glass U tube (560mm long, 10mm i.d.) filled with 100µL of pure dichloromethane (DCM), or through a 1L gas-wash bottle containing a filter paper disk (9cm diameter) to which 100µL of test solution was applied. For *G. brevipalpis* a mixture of 3*n*-propyl phenol, 1-octen-3-ol and *p*-cresol diluted in DCM at concentrations of 100ng/µL and 1µg/µL added to acetone (POCA[1] and POCA[2] respectively) at a ratio of 1:4:8:500 was used. For *G. pallidipes*, the mixture of 3*n*-propyl phenol, 1-octen-3-ol and *p*-cresol (POC) was diluted in DCM at a concentration of 100ng/µL.

More recently, tracking systems have been developed with the aim of studying the flight behaviours of insects. For example, Witzgall & Arn (1990) have shown that the flight efficiency of male European grapevine moths, *Lobesia botrana*, was reduced when attracted by the

synthetic pheromone blend compared to the response to calling females where they steered more precisely into the wind towards a mate. 2D recordings of the flight of morsitans group tsetse fly spp. have been carried out in the field and in wind tunnels. Tsetse flies respond to wind direction while flying in an odour plume (Gibson & Brady, 1985; Gibson & Brady, 1988; Colvin *et al.*, 1989; Warnes, 1990b; Gibson *et al.*, 1991) and these authors conclude that tsetse flies use chemoanemotaxis to head towards an odour source. Moreover, Colvin *et al.* (1989) confirmed in a wind tunnel study that it is the visual flow of information due to fly movement over the ground that permits optomotor anemotaxis. Moreover, these studies confirmed the occurrence of sharp turns when contact with the odour plume was lost (Gibson & Brady, 1985; Gibson & Brady, 1988; Gibson *et al.*, 1991) and also permitted to measure parameters such as the mean angles of turns (5°-386°) and ground speeds of tsetse flies (0.9-9m/s), even though there were large variations between insects (Gibson & Brady, 1985; Gibson & Brady, 1988; Colvin *et al.*, 1989). Gibson *et al.* (1991) filmed the flight behaviour of *G. pallidipes* and *G. m. morsitans* in the field in the vicinity of a visual target in the presence of an odour plume and concluded that the higher concentration of the odour plume near the target influences the flight responses of tsetse flies by increasing their circling behaviour causing them to slow down.

In comparison to the use of binary behavioural criteria mentioned above, continuous recording of flight responses provides data on quantifiable flight parameters such as speed, straightness, angular sum and angular velocity, i.e. sets of variables that are amenable to more robust statistical analyses, as used in the earlier studies cited above. This permits comparison of quantitative aspects of the flight responses of tsetse flies to treatments. Data is presented in this chapter on the responses of *G. brevipalpis* filmed in 3D to human breath presented with and without a visual target in a wind tunnel. This 3D system was only available for the last six months of this thesis research, so the behavioural data presented in the subsequent thesis chapters were recorded by eye.

Materiel & Methods

Insects

Pupae were obtained from the International Atomic Energy Agency (Vienna). Adult *G. brevipaplis* were maintained in rectangular cotton netting cages (1mm mesh, 25×15×15cm) covered with a transparent plastic bag containing wet tissue to maintain 100% RH, in an environmental chamber at 26°C, 8h light and 22°C, 10h dark, with 2h light ramps at dawn and dusk (Annex A). Unmated flies of each sex were fed bovine blood meals through a silicon membrane (Langley & Maly, 1969) at 2-3 day intervals from day 2 of emergence (Annex C). Flies tested in the wind tunnel had received at least 2 blood meals and were starved for 3-5 days (Annex B).

Wind tunnel

The climatized wind tunnel (working area: 250cm long 100×100cm) is made of non-reflecting glass. A centrifugal ventilator moves the humid- and temperature-controlled air (85±1% RH, 25±0.1°C) across the tunnel at 40cm.s^{-1} through active charcoal cartridges placed at either end of the working area. A semi-laminar air flow was produced through a perforated steel screens (1mm thick, 3mm round holes, 51% of air passage) covered with white mosquito netting (cotton, 1mm mesh) placed upwind and a laminar flow screen at the downwind end of the wind tunnel. Overhead illumination was provided by high frequency fluorescent lighting (36W, >1kHz, with 8 Philips TL-D) running the length of the tunnel in two groups of 4 tubes 20cm apart in a housing 120cm wide to provide ~700lux on the floor. The floor of the wind tunnel was covered with a medium density fibreboard (4mm thick, light brown), and the sides with white cotton sheets backed up by dark grey folded curtains forming random vertical bands along the sides of the tunnel. The light colours on the floor and the side of the wind tunnel permitted to increase the contrast between insect and background and the folded grey curtain provided lateral visual cues to the flying insects.

The plume structure was visualised by generating a plume of ammonium acetate and by measuring the CO_2 concentration at different point in the wind tunnel during breath delivery. Because of the semi-laminar air flow stream, the odour plume can be described as a large cone of ~40cm wide entering the tunnel at a height of 40cm and ~80cm wide downwind at a height of 40cm.

Experimental setup

Flies were tested individually in the wind tunnel. Each fly was transferred three to ten minutes before being tested into a plastic release cage (transparent PVC cylinders 15cm×10cm) with sliding ends covered with cotton netting (1mm mesh). The release cage was placed horizontally

on a stand at 40cm from the floor at the downwind end of the tunnel and, after fly acclimatisation, both doors were lifted slowly. If the fly did not exit the cage during opening it was successively exposed for 1 min to odour-free air (negative control) and then for 1 min to human breath.

Human breath was delivered into the wind tunnel in two different manners via an aluminium tube bent downwind at a height of 40cm from the tunnel floor that entered a plume generator. This generator stood at 35cm hight (to its center) and consisted of a stainless steel cylinder (10.5cm long, 11cm o.d.) with an aluminium tapered end (4cm long, 11cm to 14cm o.d.) closed off by a bronze grid (1mm mesh). Human breath was introduced into the wind tunnel by blowing directly through a 5mm i.d. silicon tube that was linked to the aluminium tube and the plume generator (see above). A more standardised delivery of human breath was made by sucking breath from a Tedlar® gas-sampling bag (25L, SKC, USA) with a pump (MPC 100-E, Labovac®, Saskia, Germany) and releasing it into the wind tunnel via a 2mm i.d. teflon tube linked to the aluminium tube and the plume generator (see above). The Tedlar bag had been filled with human breath at least 30 minutes before the first experiment. A polystyrene sphere (diameter 12cm) covered by a phtalogen blue cotton (reflectivity 650nm) was added for experiments where a visual stimulus was tested with human breath. The sphere stood in the middle (height 40cm, depth 50cm) or to one side (height 40cm, depth 5cm) at the upwind-end of the wind tunnel. As *G. brevipalpis* needed to be activated by an odour to induce exit from the release cage, we used human breath as a stimulus for all the experiments.

3D video recording system

Two video cameras (resolution 656×491pixels at 132 frames-per-second, model exA640-120m, eXcite, Basler, Germany), equipped with objectives (SV-M0614, C-mount, f=6mm, VS Technology, Japan) were placed 87cm above the roof of the wind tunnel at 160cm from each other. The two cameras were linked via a Gigabit Ethernet interface to a computer equipped with the specially developed software *Crow* (University of Neuchâtel) using the Halcon software library (v7.1, MVTec, Germany) that generates the 3D space. After 3D calibration, this programme registers the X, Y, and Z coordinates for successively detected points of a moving object permitting to determine the position of a flying insect within the wind tunnel at any given time to an accuracy within 5mm over 2.5m. All analyses of tsetse fly trajectories were made using the software *CrowAnalyser* (University of Neuchâtel) which permits calculation of parameters characterizing each flight (see below).

Flight track analysis

G. brevipalpis performed different types of flights during one recording session, such as successive upwind and downwind flights, or circling around the release cage before or after a directed flight. To analyse *G. brevipalpis* flight behaviours, we decided to subdivide the flights into: upwind flights where the fly flew at least 1m upwind i.e. X always increasing and starting flying 15cm either side of the Y axis, i.e. in the middle of the wind tunnel as viewed from above; downwind flights where the fly flew at least 1m downwind, i.e. X always decreasing; turning flights where the fly described a circle without progressing more than 1m along the length of the wind tunnel. The following flight parameters were calculated for directed upwind and downwind flights.

Total distance covered (*d*) was determined as the distance of the complete track namely, the sum of the distances between the successive insect positions detected (*P*).

$$d_{current} = \int_{start}^{current} P_i \quad \text{or} \quad d_{current} = \sum_{i=start}^{current-1} |P_{i+1} - P_i|$$

Ground velocity (*v*), defined as the instantaneous speed of the insect. In addition, ground velocity can be decomposed into its X, Y, and Z constituents.

$$v_{current} = \frac{\mathrm{d}(d_{current})}{\mathrm{d}(t_{current})}$$

Straightness (*r*) is the quotient of the length of a straight line between the start position and the current position of the insect, divided by the *total distance covered* by the insect, where an index of 1 indicates a perfectly straight track.

$$r_{current} = \frac{|P_{current} - P_{start}|}{d_{current}}$$

The *angular velocity* (*W*) is defined as the instantaneous turning speed with reference to the previous vector of the insect at each position.

$$W_{current} = \frac{\mathrm{d}(\Theta_{current})}{\mathrm{d}(\mathrm{t}_{\mathrm{current}})}$$

The *angular sum* corresponds to the sum of all angles made by the insect along its path from the start point to its current position.

$$angular\ sum_{current} = \sum_{i=start}^{current} \Theta_i$$

The mean general direction of each flight was ascribed a *mean unit orientation vector* in the X-Y, Y-Z and X-Z planes. These orientation vectors were in addition weighted to reflect the flight *straightness* in the particular direction, the longer the vector between 0 and 1 and -1 the straighter the flight track.

$$muov = \frac{r \times (P_{end} - P_{start})}{|P_{end} - P_{start}|}$$

Even though each of the motion sensitive cameras captured images at 132 frames per second only those that arrived synchronously from the two cameras at the *Crow* interface were used to constitute the successive points of flight. The rate of positions captured per second was approximately 20Hz.

Instantaneous measurements of the above parameters can be taken at any point during insect flight and we found that their overall progression is much more related to the position of the insect in the wind tunnel than to the time of flight. Angular velocity provides a good example to visualise this. A big increase in angular velocity occurs in all flights at 1m before the upwind end where the insect sees the screen at the upwind end of the wind tunnel, that is independent of flight time (Fig. 1). In addition, inconstancies in the number of positions recorded by the cameras per second (linked to the fact that only synchronised images are used by the *Crow* software) gave rise to flight tracks with events that the insect did not or could not have made (very sharp angles, for example).

In order to efficiently compare parameters of all the flights at given positions, an interpolation system was included in the software *CrowAnalyser* permitting to analyse the tracks as a function of time or distance. As the duration of one trajectory along the wind tunnel was very short, we estimated their overall progression to be more related to the position of the insect in the wind tunnel than to the time of flight, and so we worked with distance-based interpolation. The next problem was to define an adequate distance between successive points of interpolation. This was dictated by the length of *G. brevipalpis* (~2cm) and its minimum speed of flight (>1m/s). With these parameters in mind it is unlikely that *G. brevipalpis* can undertake any significant behavioural change within a distance of 5cm along its flight track (Fig. 1). Moreover, noise is

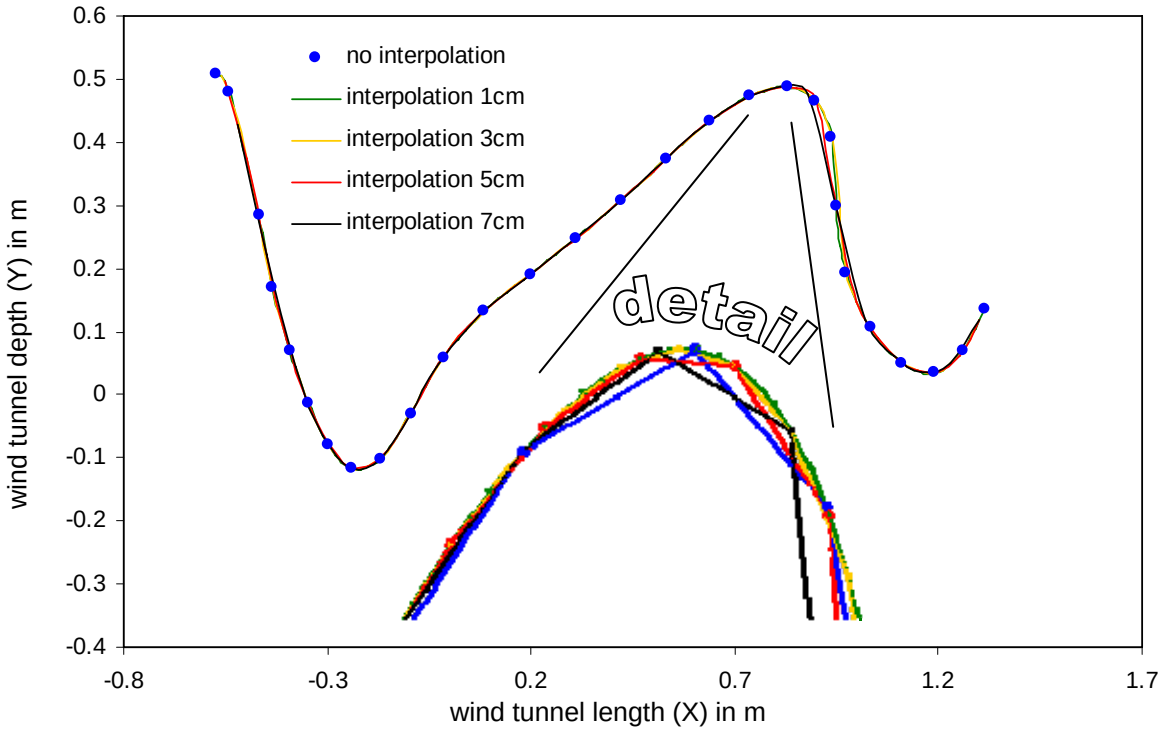


Figure 1. Upwind flight of *G. brevipalpis* interpolated at different step lengths.

At first sight the shape of the track appears similar between the original and interpolated flight, but the detailed view of the upper acute angle made by the insect shows how the representation of the curved flight is smoothened out through interpolation with increasing numbers of steps.

generated in the interpolated tracks at step lengths below 5cm (Fig. 2). The interval of 5cm chosen provides a reasonable cut-off point between the generation of non-biological movements (at longer step lengths) and noise generation (at lower ones).

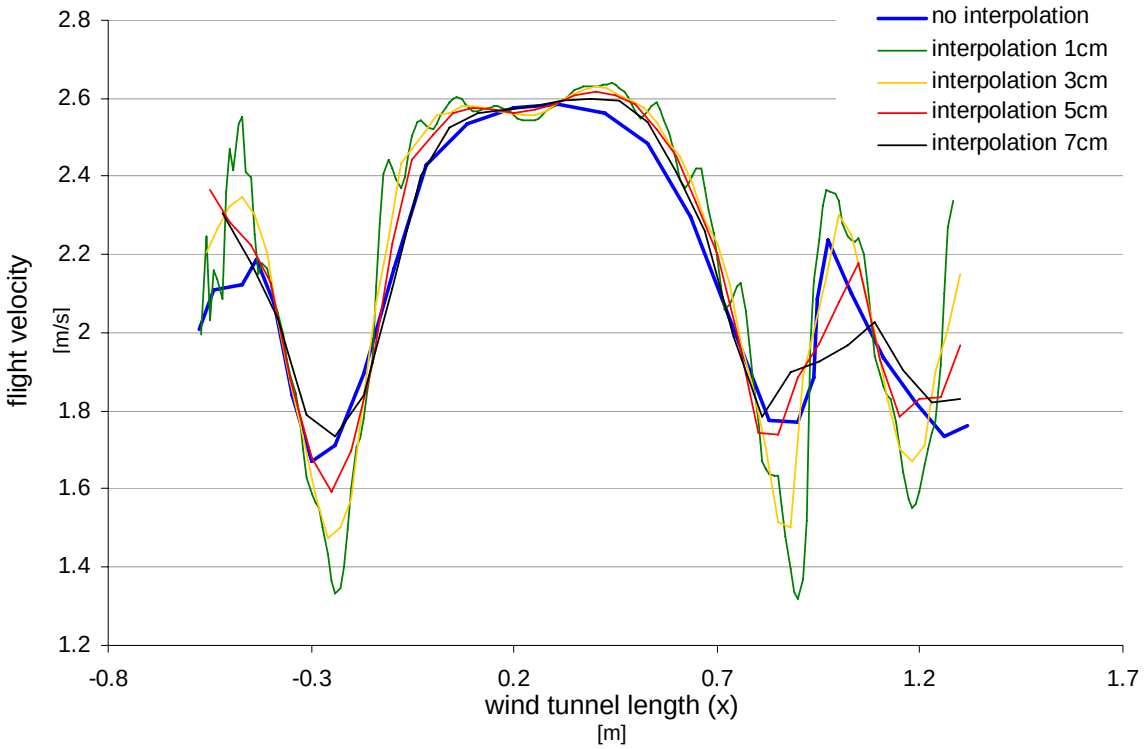


Figure 2. Progression of flight velocity without interpolation and with interpolation at different step lengths for a flight by *G. brevipalpis* along the lengths of the wind tunnel (from right to left).

Note how smaller interpolation steps can introduce "noise" on the original track and how long interpolation steps can lead to loss of biological information.

Results

G. brevipalpis flight response to a plume of human breath

The flights of 22 *G. brevipalpis* accounted for here that responded with at least one directed upwind flight to the source of human breath presented alone in the wind tunnel lasted between 3s to 141s, covering distances between 4 and 153m. The flights of a further 11 *G. brevipalpis* we are reporting on that responded to human breath in the presence of a visual target lasted between 5s to 117s, and covered distances between 6 and 40m. The flights of these flies that responded to visual and chemical stimuli were divided into three categories: upwind and downwind directed flights (Fig. 3A & B) and flights made around the visual stimulus provided by the release cage (Fig. 3C). Despite a high degree of variability between flights within the same category, detailed analysis of the trajectories permit us to ascribe the overall characteristics to each flight category and to describe flight patterns according to context.

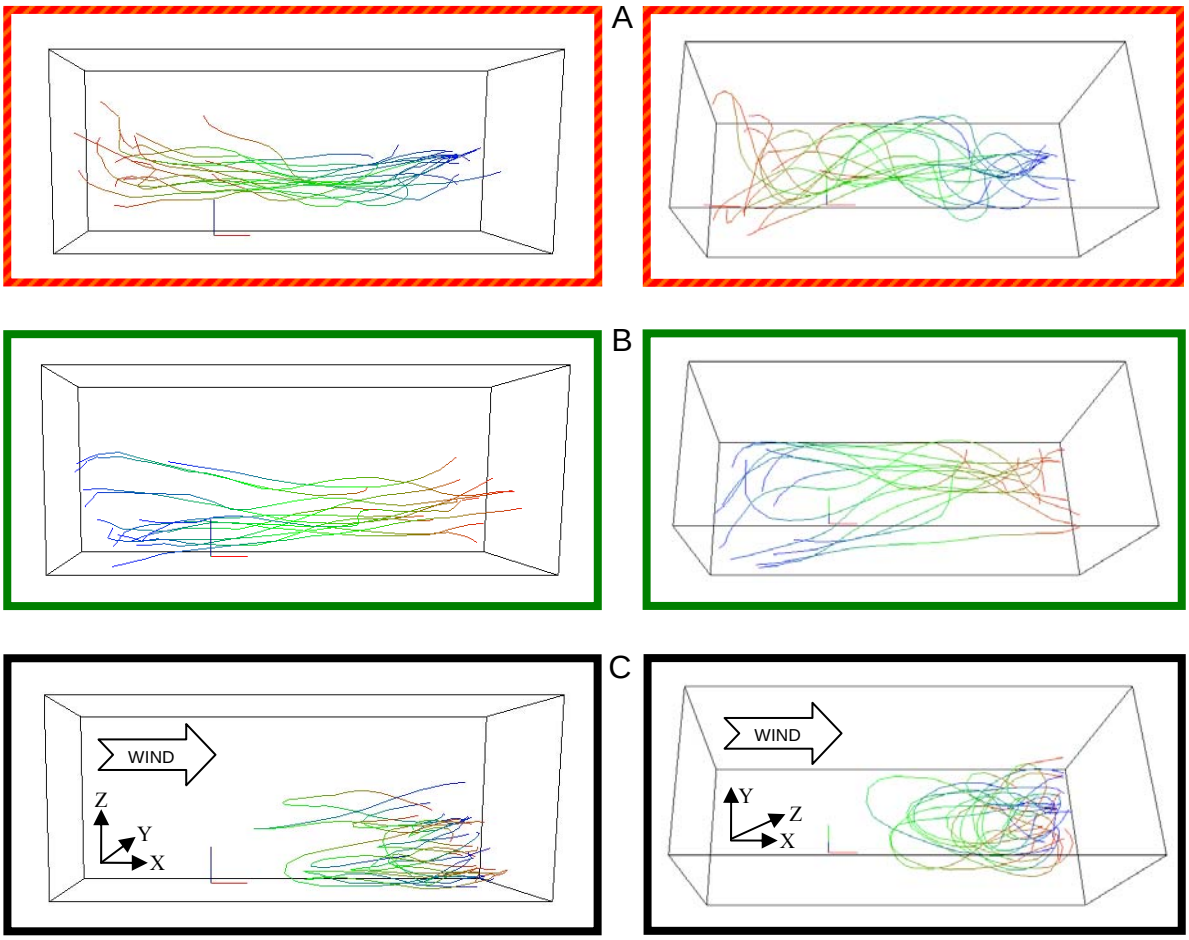


Figure 3. 3D representations of flights made by *G. brevipalpis* in response to human breath in the wind tunnel.

These flights were divided into different parts: upwind flights (A), downwind flights (B) and flights made around the visual stimulus provided by the release cage (C). The colours of the tracks provide an indication regarding flight progression ranging from blue to red; upwind flights (A) run from right to left and the downwind flights (B) from left to right. The box outlined provides a representation of the wind tunnel limits. The panels on the left are side views and on the right from a position of 30° approximately from above.

All upwind flights to breath in the absence of a visual target display a “shallow curve” pattern with a period of approximately 1m (Fig. 4). When one compares first upwind flights with other subsequent upwind flights it would appear that turns occur nearer the upwind end of the tunnel in second and subsequent upwind flights (Fig. 4).

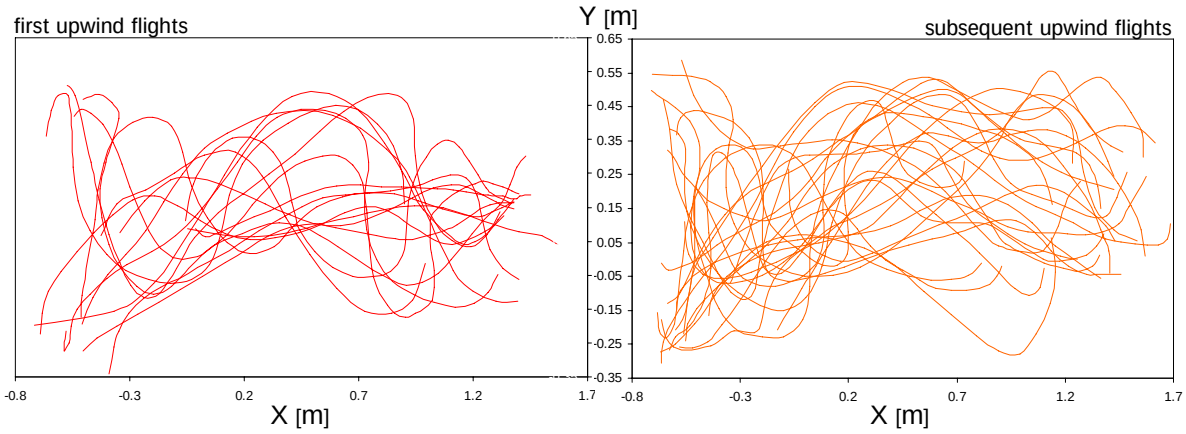


Figure 4. Top view of upwind flights of *G. brevipalpis* responding to human breath in the wind tunnel.

The X axis represents the length of the wind tunnel (2.5m) with the insect release cage placed at X=1.35 and the odour source at X=-0.8. The Y axis represents the depth of the wind tunnel (1m) with the release cage at Y=0.15 and the odour plume dispensed between Y=-0.05 and 0.35 at the upwind end. Red lines represent the track of the first upwind flights made by *G.* brevipalpis (n=16 flies) left panel and the orange lines the tracks of subsequent upwind flights (n=26) made by the same flies.

Flies leaving the release cage in response to human breath did so at a height of Z=0.35 and maintained this flight altitude (Z_{mean}=0.34±0.1) during their upwind progression; subsequent upwind flights were made at lower altitudes (Z_{mean}=0.15±0.1 ; Fig. 5).

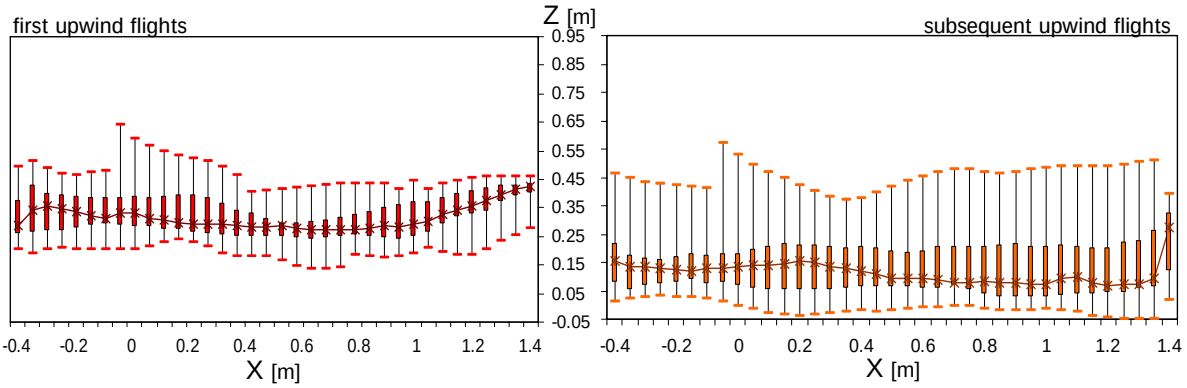


Figure 5. Side views of interpolated upwind flights made by *G. brevipalpis* stimulated with human breath in the wind tunnel.

The X axis represents the length of the wind tunnel (2.5m) with the insect release cage placed at X=1.35 and the odour source at X=-0.8. The Z axis represent the height of the wind tunnel (1m) with the release cage at Z=0.35 and the plume of breath dispensed between Z=0.15 and 0.55 at the upwind end. Each box plot represents the position of the median, minimum, maximum and the 25th - 75th quartiles of all tracks interpolated on X every 5cm. The box plots in red are the altitudes of the first upwind flights made by the flies (n=16 flies ; left panel) and the box plots in orange are the flight altitudes of subsequent upwind flights (n=26).

The maximum speed of *G. brevipalpis* flights in the wind tunnel regularly reached ≈3m/s with no real differences between first and subsequent upwind flight speeds (Fig. 6). The mean instantaneous speeds reached along the length of the wind tunnel was similar for all upwind flights, increasing to ≈2.4m/s at 1m from the upwind end, and then decreasing. The lower speeds recorded for the first upwind flights in the initial 80cm of the wind tunnel were due to the departure of the flies from the release cage.

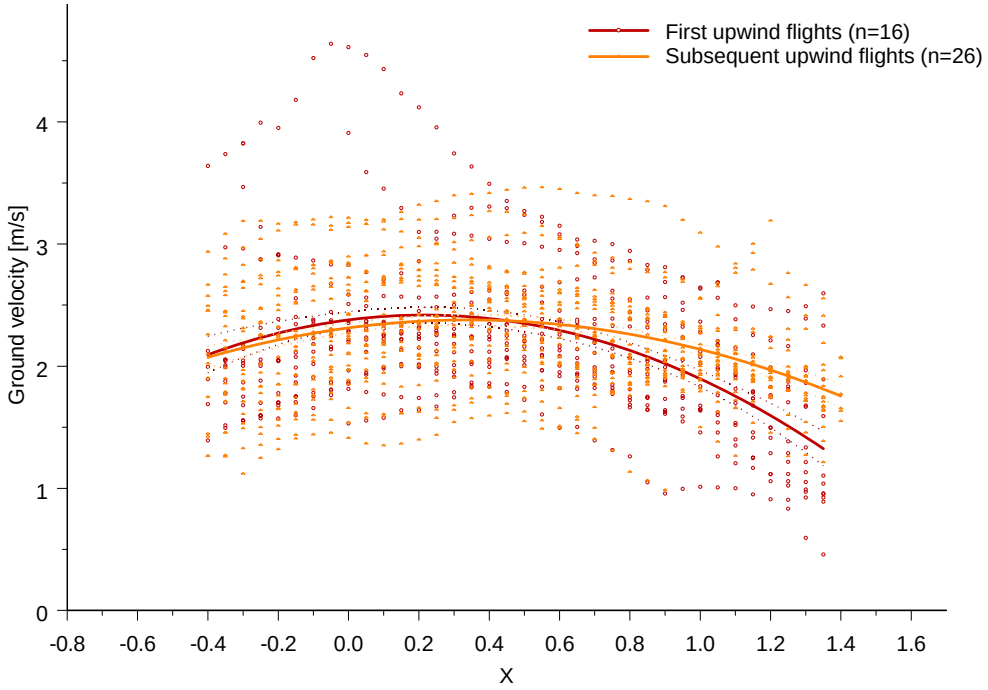


Figure 6. *G. brevipalpis* instantaneous ground speeds extrapolated at successive 5cm positions along the length of the wind tunnel (X) during first upwind and subsequent upwind flights when stimulated by human breath.

The insect release cage was placed at X=1.35 and the odour source at X=-0.8. The coloured lines are polynomial regressions for the two categories of upwind flights.

When *G. brevipalpis* responding to human breath arrived at the upwind end of the wind tunnel they most frequently returned downwind. A comparison between the first upwind and the first downwind flights showed that these flights were different. Flies flying upwind did so at a medium altitude of Z=0.35 and depth of Y=0.10, i.e. in the middle of the wind tunnel as seen from above. By contrast, most downwind flights started from the sides (0>Y<0.3) and from an altitude nearer the floor of the wind tunnel (Z=0.1 ; Fig. 7). Most of these downwind flights terminated in the middle of the wind tunnel depth (Y=0.2) and at a height just below the release cage (Z=0.3).

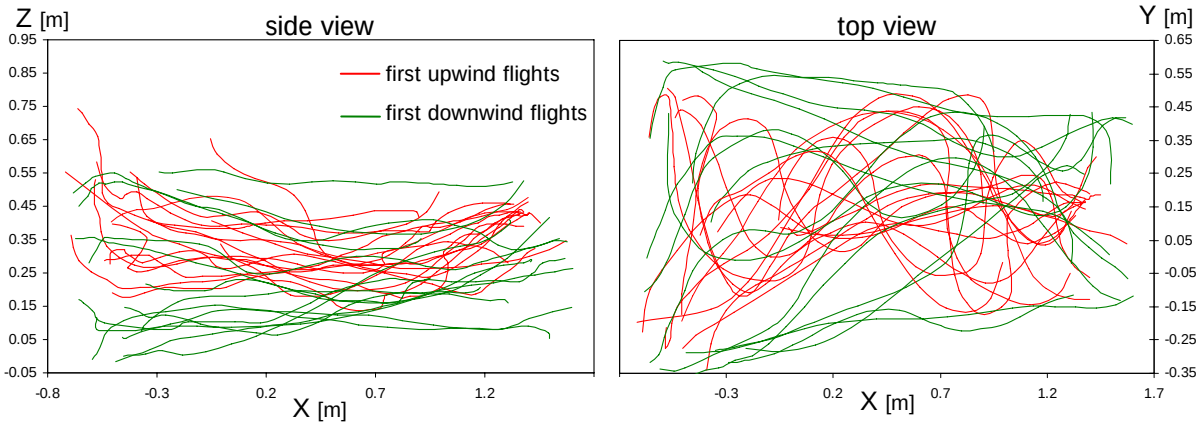


Figure 7. Top and side views of first upwind and first downwind flights made by *G. brevipalpis* stimulated by human breath in the wind tunnel.

The red lines are the first upwind flights (n=16) and the green ones correspond to the first downwind flights (n=15). Note that the upwind flights direction are measured by a decrease along X, whereas the downwind flights are measured by an increase along X. For more details about the positions of the plume of breath and insect release cage see legends to *Figures 4* & *5*.

First downwind flights by *G. brevipalpis* were straighter (r_{mean}=0.90±0.07) compared to the first upwind flights (r_{mean}=0.79±0.11 ; Fig. 8) probably due to the visual stimulus provided by the release cage as the flies flew downwind. Moreover, the variation associated with flight straightness indices was much higher during upwind than downwind flights.

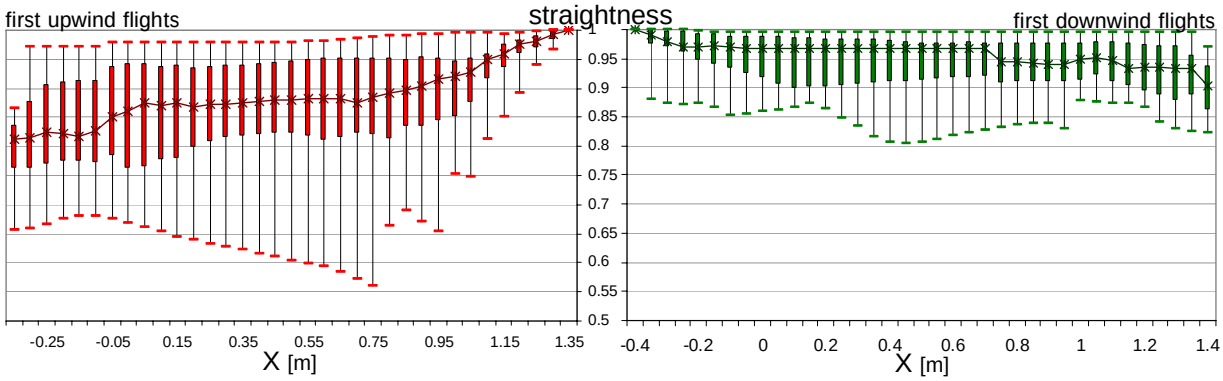


Figure 8. Instantaneous straightness indices for first upwind (left) and first downwind flights (right) made by *G. brevipalpis* in response to breath in the wind tunnel extrapolated at successive 5cm positions along the length (X) of the wind tunnel.

The box plots in red (left panels) are the first upwind flights (n=16 flies) and the green ones (right panels) are the first downwind flights (n=15). For more details regarding the box plot representation see legend to *Figure 5*.

The angular sum associated with downwind and first upwind flights climbed during tsetse fly movement along the length of the wind tunnel. This increase was more regular and less variable during downwind flights (Fig. 9). The final angular sum_{mean} reached was 46°±23° for first upwind flights compared to an angular sum_{mean} of 20°±8° for the first downwind flights.

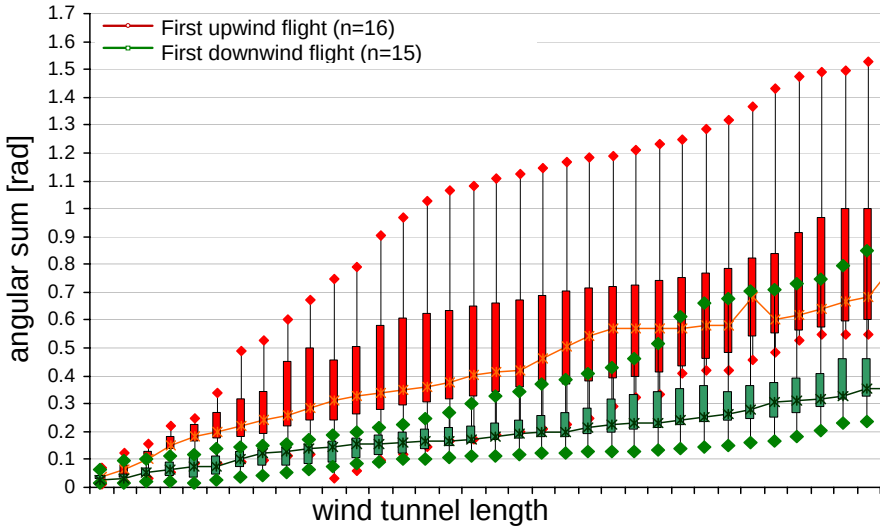


Figure 9. Progression of the angular sum extrapolated at successive 5cm positions along the length of the wind tunnel for first upwind and first downwind flights of *G. brevipalpis* responding to human breath

The box plots in red correspond to the first upwind flights without a target (n=16 flies) and the ones in green correspond to the first downwind flights (n=15). Note how the box plot that covers the 25th to 75th percentiles is shorter in the first downwind flights.

Flight response to a visual target in a plume of human breath

The above experiments were made without any visual target, except for the release cage which may have been used as a landmark during the downwind flights by *G. brevipalpis*. The addition of the blue sphere at the upwind end of the wind tunnel permitted to compare directed flight by *G. brevipalpis* using olfactory information alone with the situation where the fly could employ a combination of olfactory and visual cues. In the absence of stimulation with human breath no flies left the release cage even with the blue target placed 2.15m upwind. In the presence of breath all flies exiting the cage flew directly to the blue target. Only 1 in 11 of these flies made a downwind flight, whereas without the target 15 of the 16 flies responding to breath with an upwind flight made at least one downwind flight. First upwind flights to the target were confined to the middle of the wind tunnel (Y_{mean}=0.17±0.08), directed to the blue sphere at Y=0.10 with low variations in the horizontal and vertical planes between flights (Fig. 10). On the contrary, the first upwind flights to breath without the blue target showed more variations in the horizontal plane as the tsetse flies advanced along the length of the wind tunnel.

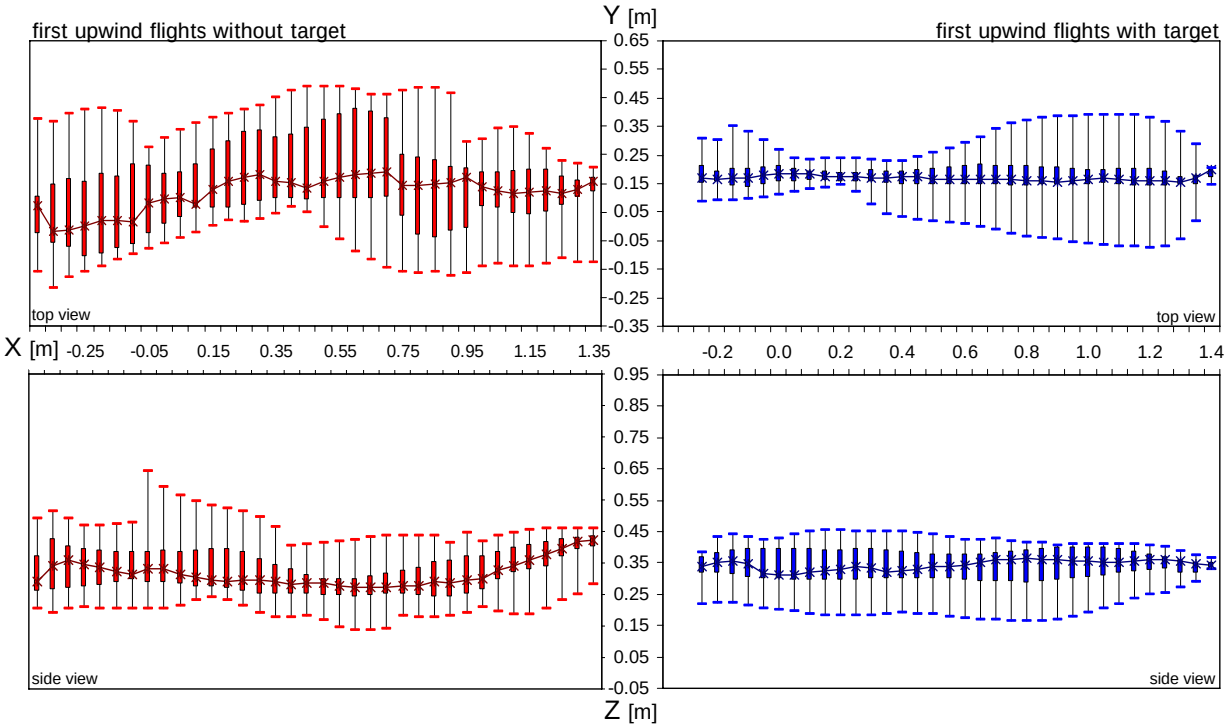


Figure 10. Top (upper) and side (lower) views of deviations in the XY and XZ planes for first upwind flights made by *G. brevipalpis* stimulated by breath extrapolated at successive 5cm positions along the length (X) of the wind tunnel with (left) or without (right) the presence of a target.

The box plots in red correspond to the first upwind flights without a target (n=16 flies) and the ones in blue correspond to the first upwind flights with a target (n=11). Note how the box plot that covers the 25th to 75th percentiles is very short in the XY plane for upwind flights with the target present. For more details regarding the axes and position of the plume of breath see legends to *Figures 4* & *5*.

These observations were confirmed by the indices of straightness for first upwind flights with and without the target: the mean straightness index for first upwind flights to the target was 0.97±0.04 compared to a value of 0.79±0.11 without the target. Variations between instantaneous flight straightness values were much higher in the absence of the visual target (Fig. 11).

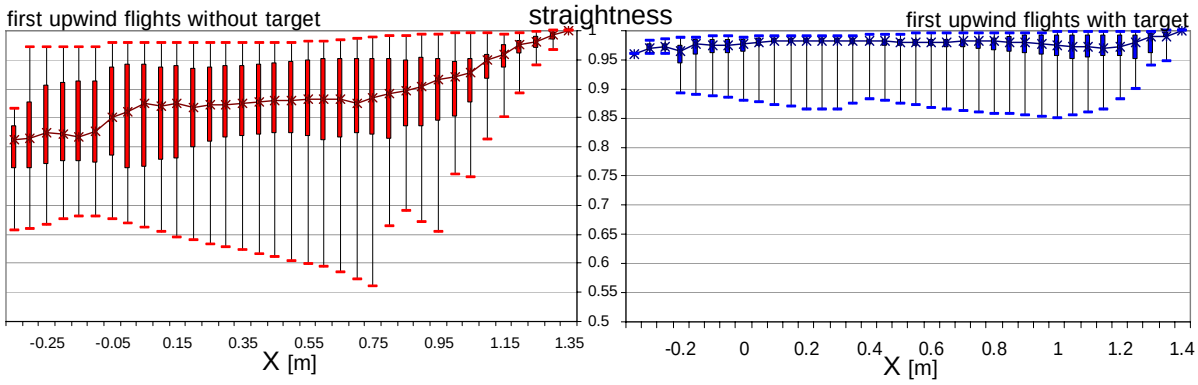


Figure 11. Comparison of the instantaneous straightness indices for first upwind flights made by *G. brevipalpis* stimulated by breath extrapolated at successive 5cm positions along the length (X) of the wind tunnel with (left) and without (right) the visual target at the upwind end.

For more details regarding the box plot representation see legend to *Figure 5*.

Measures of the instantaneous angular sum followed a similar progression along the length of the wind tunnel for tsetse responding to breath either with or without the visual target with an increase in the angular sum as the flights progressed (Fig. 12). However, the angular sum was overall lower in the presence of the blue target giving an angular sum_{mean} of 23°±11° with the blue target at the upwind end of the wind tunnel compared to a final angular sum_{mean} of 46°±23° without the target.

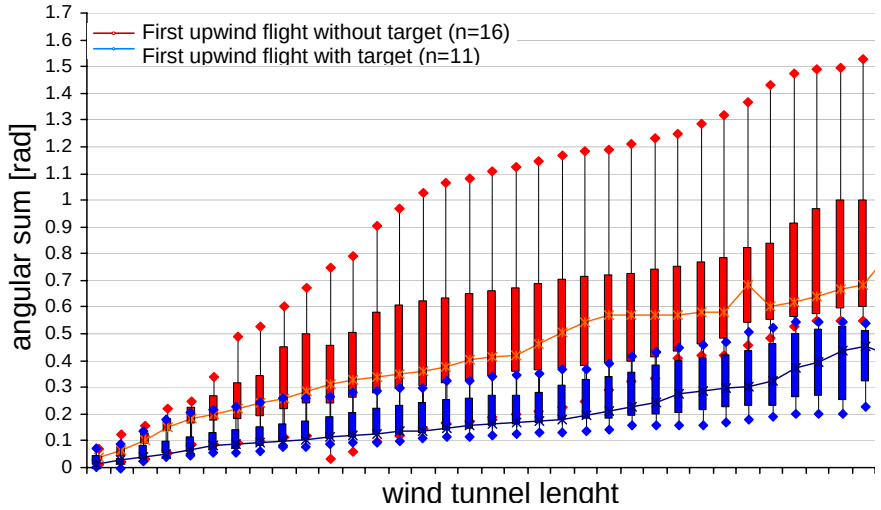


Figure 12. Instantaneous values of the angular sum extrapolated at successive 5cm positions along the length of the wind tunnel for first upwind flights by *G. brevipalpis* responding to human breath without (red) and with (blue) a visual target in the plume at the upwind end of the wind tunnel.
For more details regarding the graph see legend to *Figure 9*.

Another interesting parameter to examine was the manner in which speed was modified in flights with and without the visual target (Fig. 13). With or without the target, the speed of the upwind flights increased until 1m before the upwind end of the wind tunnel (X=0.2). However, the mean maximum speed reached at this point in the presence of the blue sphere was only ≈2m/s in comparison to ≈2.4m/s for the flights in the presence of breath but without the visual target.

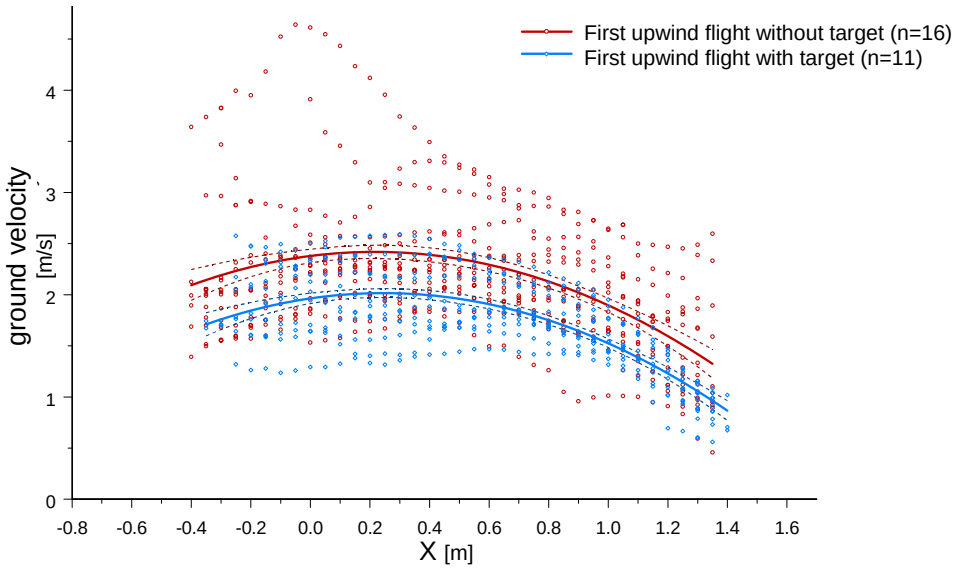


Figure 13. Instantaneous ground speeds of *G. brevipalpis* responding to human breath extrapolated at successive 5cm positions along the length (X) of the wind tunnel for first upwind flights without (red) and with (blue) a visual target in the plume at the upwind end of the wind tunnel.
For more details see legend to *Figure 6*.

Comparisons between the flight categories can be made using the mean unit orientation vectors, a parameter that provides an estimate of flight "efficiency" (Fig. 14). On the XY and XZ planes, all the flights were centred on the Y and Z=0 axes, demonstrating a progression mostly along the X axis. On the XY plane (flights seen from above ; Fig. 11), the most variable fligths were those made downwind (mean angular variation ±10°) followed by upwind flights subsequent to the first in the absence of the visual target (mean angular variation ±8°). First upwind flights without the visual target showed a similar orientation vector variation (mean angular variation ±7°). The first upwind flights in the presence of the visual target were all highly directed to the target (mean angular variation ±3°). Similar conclusions can be drawn for the XZ plane (flights seen from the side ; Fig. 11) with unit orientation vectors of first, subsequent upwind and first downwind flights without target (mean angular variation of ±5°, ±6° and ±4°, respectively) more dispersed than first upwind flights to the target (mean angular variation ±2°).

The predominating role of the visual target was demonstrated by placing the blue sphere in the upwind end of the wind tunnel but to one side (X=-0.65, Y=-0.25 and Z=0.35). In this situation, flies (n=4) that left the release cage subsequent to stimulation with human breath flew directly to the blue sphere (Fig. 15).

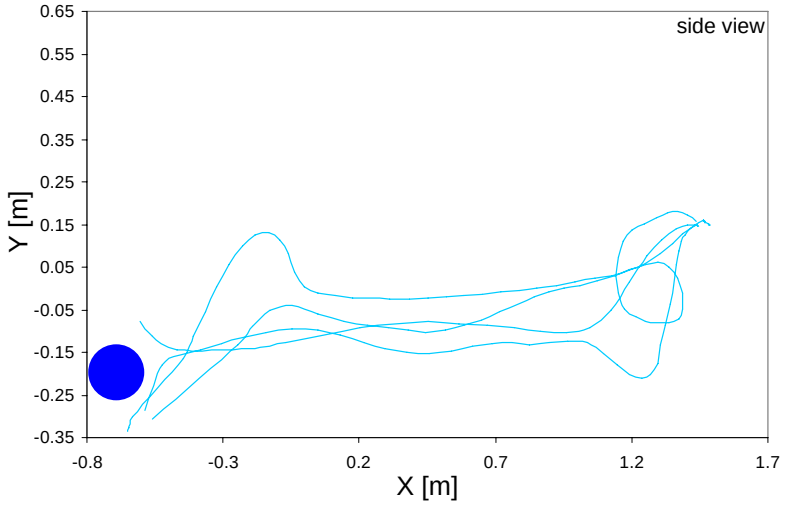


Figure 15. Top view of the first upwind flights made by *G. brevipalpis* responding to human breath with a visual target at an upwind end of the wind tunnel but placed to one side (X=-0.65, Y=-0.25 and Z=0.35). The plume of breath enter the wind tunnel between Y=-0.05 and 0.35

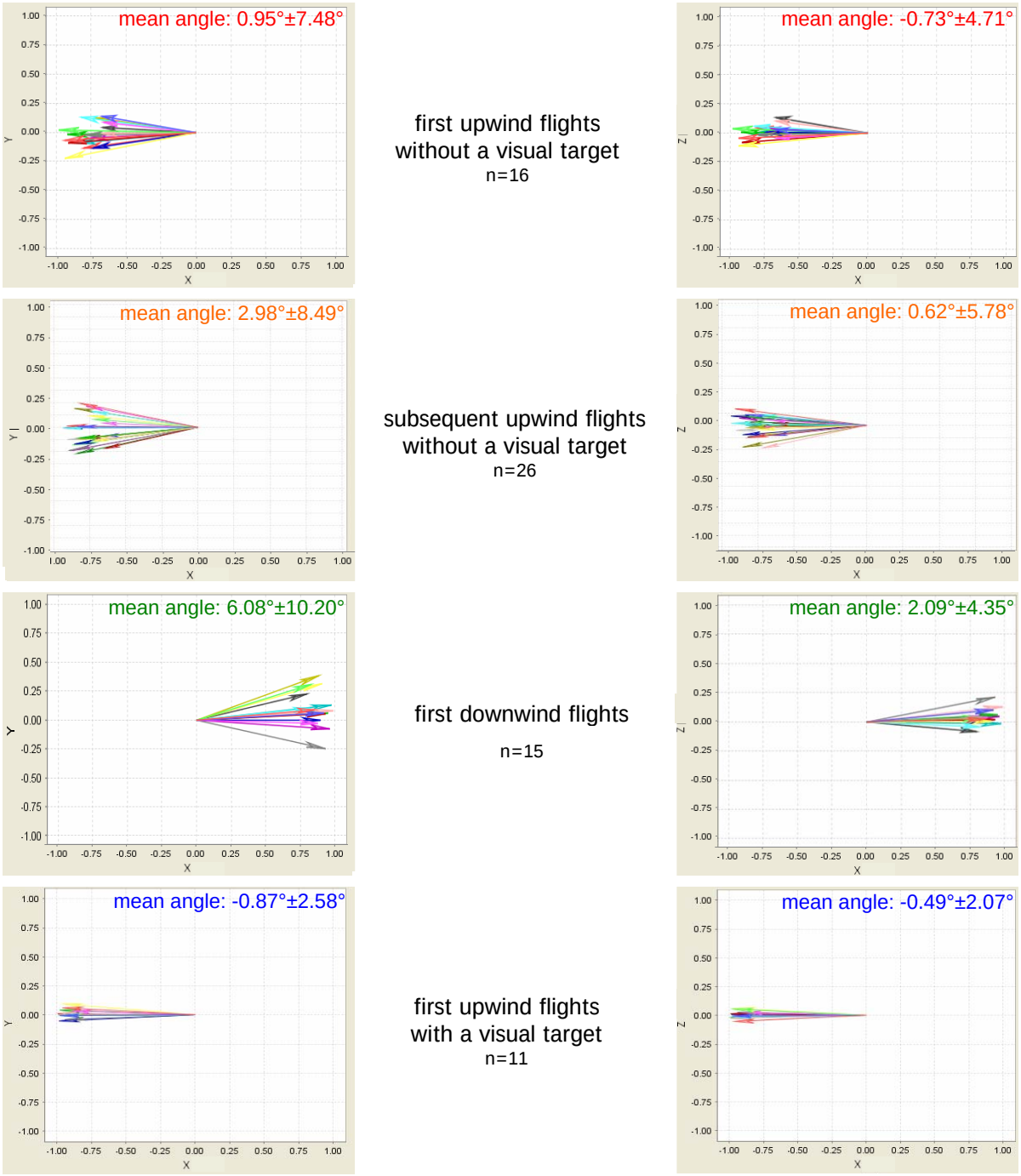


Figure 14. Mean unit orientation vectors in the XY and XZ planes for *G. brevipalpis* flights in response to human breath with and without a visual target in the wind tunnel.

The axes of each panel depict to the length (X), height (Z) and the depth (Y) of the wind tunnel. Each arrow represents the orientation vector of one tsetse fly flight, and the arrow length corresponds to the straightness of the flight (represented 0 to 1 and -1 on the X, Y and Z axes).

Discussion

The odour plume of human breath was situated in the lower part of our wind-tunnel (up to 70cm high). All the upwind flights made by *G. brevipalpis* were situated below 70cm of altitude. Similar heights of flight were reported in the field by Bursell (1984b), Gibson & Brady (1988) and Torr (1988a; 1988b). Bursell (1990) suggested that this flight altitude allows tsetse flies to encounter the lower edge of the odour plume and so to successively encounter pockets of air with and without the host emanation. In our wind tunnel experiments we can assume that *G. brevipalpis* flew mostly inside the plume heading towards the odour source using chemoanemotaxis. The flies tended to fly upwind at a lower altitude in subsequent upwind flights, suggesting an effort on their part to use the lower edge of the plume. As tracking *G. brevipalpis* flights was made in 3D here we were able to measure the flight altitude variations. This is an important parameter with regard to flight energetic costs. We did not observe much variation in flight heights for any of the directed upwind or downwind flights recorded, with the exception of the parts of the downwind flights where the flies climbed to a height beneath the release cage. This demonstrates an efficiency in tsetse flight where horizontal casting is used instead of the more costly vertical shifts.

Other authors working on tsetse fly flight studied flight direction and the form of the flight path. These studies described the behaviour of tsetse flies just after entering or leaving an odour plume while flying either upwind, downwind or crosswind. Corrections in flight after such encounters are characterized by large flight loops which are not amenable to study within the confines of the wind tunnel. What can be achieved in the wind tunnel are measures of flight efficacy to different treatments. Two flight types have been described in both wind tunnels and field studies. The turning flights described by Gibson & Brady (1988) and Packer & Warnes (1991) occur mostly around a target. Such flights were recorded around the release cage or around the blue sphere in this study. These flights were not quantified here since tracking is less accurate at the extremes of the overlapping fields of view of the cameras, and the visual target and cage frequently hid the fly from view. However, what was recorded indicates a local search around a landmark. The study of what happens there may provide some indications regarding how tsetse flies behave in the vicinity of traps and permit improvement in the efficiency of their design to capture approaching flies. As previous studies were made in 2D from above it was not possible to observe and measure these flight characteristics. The second distinctive flight type covers the shallow curved directed flights already described by Gibson *et al.* (1991) in a field study. These are flights with a straightness index higher than 0.55. Such flights were recorded here for *G. brevipalpis* for successive upwind and downwind flights by the same fly. This was already recorded for *G. m. morsitans* in a wind tunnel (Bursell, 1990). None of 68 directed flights we

selected for analysis here had a straightness index lower than 0.55. This type of flight permits the tsetse to progress to a host.

The effectiveness of a search strategy can, in addition to straightness, be assessed with parameters such as flight speed and turning rate as undertaken by Wiliams (1994). The ground speed of morsitans group tsetse species recorded in the field was always higher than recorded in wind tunnel experiments (Gibson & Brady, 1988; Colvin *et al.*, 1989; Warnes, 1990b; Gibson *et al.*, 1991). Colvin *et al.* (1989) and Warnes (1990b) measured a mean ground velocity of between 1-2m/s for *G. m. morsitans* and *G. pallidipes* in their wind tunnel, whereas Gibson & Brady (1988) and Gibson *et al.* (1991) measured mean ground velocities of between 5-7m/s for the same tsetse flies species in the field. Nevertheless, Colvin *et al.* (1989) measured a speed of 3.5m/s±0.9 for the same tsetse spp. that had taken off 1m before the point of measurement in the field, suggesting that tsetse flight speed recorded in the wind tunnel may not reflect what the species can achieve due to the limited space. Here, the ground speed (1.5-3m/s) reached by *G. brevipalpis* is higher than in previous wind tunnel studies with other species. However *G. brevipalpis* is the biggest tsetse species, a strong flyer and our wind tunnel is longer than those used in the studies cited above. The ground speed decrease recorded at the upwind end of the wind tunnel for *G. brevipalpis* in this study is probably due to the mechanical and aerodynamic needs of turning as already noted for *G. pallidipes* and *G. m. morsitans* near visual targets by Gibson *et al.* (1991).

Despite the fact that upwind and downwind flights by *G. brevipalpis* were characterised by similar shallow curves, the measures of the instantaneous angular sum did not follow a similar pattern along the length of the wind tunnel. During upwind flights, either with or without the visual target, the variation in angular sum among the tsetse fly flights was high compared to variations among downwind flights. Since the angular sum evolution between first upwind flights with a target was similar to first downwind flights, *G. brevipalpis* probably used the release cage as a visual target that induced straighter downwind flights. Warnes (1990b) indicated that the sinuosity of tracks described by *G. m. morsitans* and *G. pallidipes* increased in proportion to CO_2 concentration, so we may suggest that during their upwind flights in this study, the *G. brevipalpis* trajectories were not just modulated by the visual target but also by the plume of human breath that increased in concentration as they progressed up the wind tunnel.

The most notable aspects of the flights recorded for *G. brevipalpis* here are the more directed and slower flights to the visual target placed in the plume of breath. This flight pattern to an attractive source of stimuli is rather counterintuitive as one would expect faster flights. Apparently *G. brevipalpis* controls both the direction and speed of flight within stricter limits in the presence of both visual and chemical cues in an effort to increase the efficiency of approach, just as required for landing by birds and aeroplanes. Remarkably the same phenomenon of

slower more directed orientation to human breath was already recorded for the tropical bond tick, *Amblyomma variegatum* walking on the ground (McMahon & Guerin, 2002).

We can conclude from the above that *G. brevipalpis* possesses the ability to hold flight parameters under stricter control in the presence of both chemical and visual stimuli. The predominating role of the visual component was confirmed by the directed flights by *G. brevipalpis* to the target placed outside of the plume of breath. However, the turning rate during directed flights is probably influenced by odour. This shows that tsetse flies are capable of complex behaviours during host searching with a high degree of interaction between their visual and olfactory sensory inputs.

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Chapter 2

Olfactory and behavioural responses of *Glossina* spp. to the rumen metabolites

Abstract

Herbivores are the preferred hosts for tsetse flies and among them ruminants occupy a dominant position. Volatile chemostimuli from the rumen fluid of cattle are regularly eructated into the atmosphere permitting these metabolites to be used as cues by tsetse flies searching for a blood meal.

We performed a fractionation of bovid rumen fluid and analysed in detail tsetse fly responses to the predominant compounds present, i.e. carboxylic acids. Using antennae of three different tsetse fly species as biological detector coupled to a gas chromatography we were able to quantify the detection threshold for different carboxylic acids in rumen fluid.

The flight responses of *G. pallidipes* in a wind tunnel were stronger using a mixture of butanoic, pentanoic, hexanoic and isovaleric acids at a ratio similar to that present in rumen fluid than to an equimolar ratio of these carboxylic acids. Carboxylic acids added singly to acetone did not significantly change the effect of acetone on its own on the behavioural responses of *G. brevipalpis* in the wind tunnel with the exception of isobutanoic, hexanoic and cyclo hexane carboxylic acids that moderately increased some behaviour criteria.

Key words : tsetse flies, olfaction, rumen, electroantennogram recordings, wind tunnel, carboxylic acids.

Introduction

Tsetse flies (*Glossina* spp.) locate hosts beyond their visual range by following olfactory cues. Their olfactory perception extends to 60-120m downwind of an odour (Vale 1977) and visual cues, including contrast, shape and colour are important in host location at a shorter range of 10m (Gibson & Torr, 1999; Vale, 1974). A variety of trap designs have been developed for tsetse flies (Vale 1974), all based on the shape perceived and colour discrimination of tsetse flies, and on the contrast between the trap and the background vegetation (reviewed by Green (1994)). A significant advance came with the development of odour baits that can dramatically increase the attractiveness of these devices and improve trap catches by several fold. The major olfactory stimulants used nowadays in the traps for tsetse flies are acetone, 1-octen-3-ol, 3*n*-propyl phenol and *p*-cresol, originating from odours of oxen (Bursell et al., 1988; Hall et al., 1984; Hassanali et al., 1986; Vale & Hall, 1985).

Herbivores are among the preferred hosts for many haematophagous insects due to their habit of herding and their sessile nature. Ruminants, a big group of wild and domesticated herbivore mammals, are found in varied geographical regions and are successfully exploited by tsetse flies. In the rumen, the 1st chamber of their forestomach, a variety of micro-organisms hydrolyses plant polymers and ferment monomers to yield energy. These products formed in the foregut are continually evacuated through eructation with the fermentation gases and may provide tsetse flies with the appropriate blend of chemostimuli to track ruminants.

Research to date on the sensory ecology of tsetse flies has been sparse, concentrating on one or a few product families, and currently used odour attractants do not increase the trapping of most of the *palpalis* and *fusca* spp. (Mwangelwa *et al.*, 1991; FAO, 1992; Green, 1994). We therefore decided to investigate on the end products synthesised by microbes in the rumen of the cow in an effort to identify new chemostimuli that could permit improvement of lures for tsetse flies.

Materiel & Methods

Insects

Pupae were obtained from international atomic energy agency (IAEA, Vienna) and were kept until emergence in plastic boxes (28×9×8cm). Adults of *Glossina pallidipes*, *G. fuscipes* and *G. brevipaplis* were maintained in rectangular cotton netting cages (1mm mesh, 25×15×15cm). They were covered by a transparent plastic bag containing a wet tissue to keep 100% of relative humidity (RH), and held in an environmental chamber at 26°C, 8h light and 22°C, 10h dark, with 2h light ramps at dawn and dusk (Annex A). Unmated flies of each sex took blood meals at 2–3 days interval from day 2 of emergence.

Flies used for electrophysiological recordings were unmated and unfed flies of 2 to 4 days old were kept at high humidity. Flies tested in the wind tunnel were used after at least 2 blood meals and starved for 3-5 days (Annexes B & C). They were kept during experiments in the environmental chamber (25°C, 65% RH) housing the wind tunnel.

Rumen collection and fractionation

Rumen fluids were collected from the abattoir (La Chaux-de-Fonds, CH) from freshly slaughtered cows by squeezing the bolus, and prepared the same day (Fig. 1). After

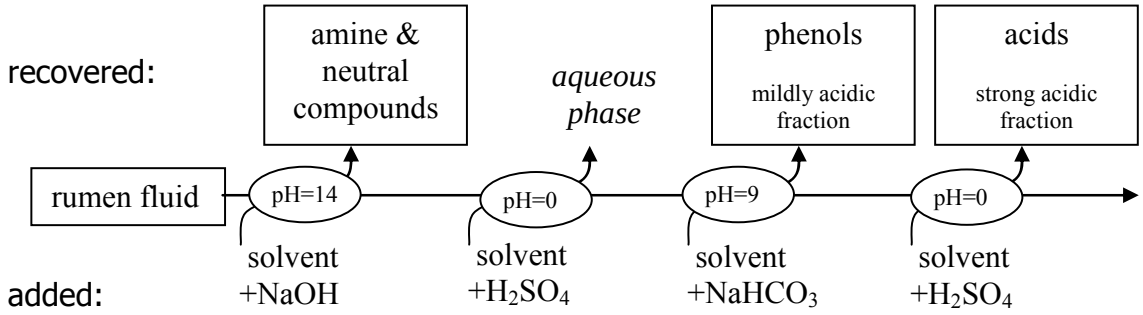

Figure 1. Scheme of the fractionation of rumen fluid using pH modification.

Rumen fluid was collected from the abattoir from freshly slaughtered cows by squeezing the bolus. This fluid is a rich mixture of different compounds which can be separated by pH modification into different fractions.

centrifugation (30min at 3600R/min), the rumen supernatant was brought from pH 5-6 to pH 14 by addition of 10% NaOH. At this pH amines and neutral molecules can be extracted into dichloromethane (DCM, Merck, analytical grade). This first step in the extraction was repeated twice using the same solvent with the aim to increase the yield of amines and neutral compounds. After this, the rumen fluid was brought to pH 0 with 20% H_2SO_4 and the remaining organic compounds extracted into DCM. This organic fraction was then washed with $NaHCO_3$ solution (pH 9) so that phenols in the non ionised state were retained in the organic phase whereas carboxylic acids were recovered in the aqueous phase. These acids were subsequently extracted into DCM after acidification as above. Yields of the phenolic and acidic fractions were

satisfactory, but the yield in the amine and neutral fraction was low. In order to increase the amounts of solute on the gas chromatographic column employed to analyse the fractions (see annex E) a large volume injection technique and a long retention gap were used. The large volume injection technique consists in injecting the sample inside a glass insert in the on-column injector that is filled with glass wool and cooled (30°C). By fully opening the split valve of the injector during the injection the solvent is flushed away by the carrier gas, whereas solutes are trapped in the glass liner (Grob 1988). Subsequent closing of the split valve and heating the injector (to 230°C at 12°C/s) permits transfer of the compounds to the gas chromatographic column. A long retention gap (10m deactivated fused silica, 0.320mm i.d., BGB, Switzerland) was added between the on-column injector and the chromatographic column.

Sensory physiology

The electroantennogram (EAG) introduced by Schneider (1957) allows the measurement of the electrophysiological response of insect antennal receptor cells to stimulation with an odour. Electroantennograms from *Glossina* spp. antennae were recorded as described in Guerin & Visser (1980). The tsetse fly head was placed between two glass capillary electrodes (2mm o.d.) filled with a 0.1M KCl solution, with the tips of the electrodes placed at the base and the tip of the antenna with micro manipulators. The antenna was held in a humidified charcoal-filtered air stream (90-100% RH, 23±2°C) delivered at 1m.sec^{-1} via a glass water-jacketed tube (7mm i.d.) whose outlet was about 1cm from the preparation. The antenna preparation was connected to a computer via a high impedance preamplifier (Syntech, NL), a DC amplifier (UN-03, Syntech, NL) and an analog-digital converter (USB-IDAC box, Syntech, NL).

Coupling the antenna as a biological detector (EAD) to the effluent of a high resolution gas chromatographic column (GC) permitted the localisation of active components from the different fractions of rumen fluid (GC-EAD). In this manner the insect antenna was used as an on-line detector in parallel with the flame ionisation detector of the gas chromatograph (5300, Carlo Erba Instruments, Italy) whose trace was recorded on a computer through the same analog-digital converter (USB-IDAC box, Syntech, NL). Because we were mostly interested by the acidic fraction of the rumen fluid, we conducted separation on a polar free fatty acid phase especially designed for the separation of fatty acids (30m, 0.250mm i.d., FFAP, BGB, Switzerland).

In order to compensate for the drop in antennal response with time, the antennal preparation was regularly subjected to a pulse of odour as described in Guerenstein & Guerin (2001). 1mL of charcoal-filtered air passed through a 5mL polypropylene syringe (BD Plastipak™, Spain)

containing a filter paper strip (0.8cm×3cm) impregnated with 10µL of a solution containing 1-octen-3-ol (50:50 R/S racemic, >97% pure, Merck) at 100ng/µL in DCM.

The electrophysiological potential generated when the stimulus eluted from the GC column into the humid air stream flowing over the antenna was quantified and normalised using the equation:

$$d_x + \left(\left(\frac{d_{end} - d_{start}}{t_{end} - t_{start}} \right) \times (t_x - t_{start}) \right)$$

with d = size of the depolarisation, t = time of the depolarization, X = compound tested and start and end = pulse of 1-octen-3-ol at the beginning and at the end of the GC recording.

The responses of *G. pallidipes*, *G. fuscipes* and *G. brevipaplis* to carboxylic acids present in the rumen were compared by GC-EAD to synthetic carboxylic acids (Tab. 1).

Table 1. Synthetic carboxylic acid solutions used in GC-EAD injections and behavioural experiments

	ethanoic acid	propanoic acid	isobutanoic acid	butanoic acid	isovaleric acid	valeric acid	hexanoic acid	heptanoic acid	cyclo hexane carboxylic acid
purity	99%	99.5%	98%	99.5%	98%	99%	98%	99%	98%
supplier	Merck	Fluka	Merck	Fluka	Merck	Fluka	Fluka	Fluka	Acros

Behavioural experiments

The wind tunnel (170cm long 60×60cm) is constructed of non-reflecting glass. Two centrifugal ventilators at either end operate simultaneously to move the air across the tunnel at 30cm.s^{-1} through active charcoal cartridges and semi-laminar perforated metal screens (steer 1mm thick, 3mm round holes, 51% of air passage). Overhead illumination is provided by high frequency fluorescent lights (36W, >1kHz, Philips) running the length of the tunnel (~300lux on the floor). On the tunnel floor a brown paper sheet with a pale blue line (6.5cm width) in the middle and under the tunnel roof a white sheet with 35 black rectangular patches (4×8cm) fixed at random were used as optomotor cues. The wind tunnel sides were rendered uniformly white by white cotton curtains.

For *G. pallidipes* experiments, odour-free air at 0.5L.min^{-1} passed through a 1L gas-wash bottle in which 100µL of the tested mixture were applied on a filter paper disk (diameter 9cm). The mixtures tested were a blend of butanoic, pentanoic, hexanoic and isovaleric acids, diluted at 100ng/µL in DCM, at 2 different ratios: equimolar 1:1:1:1 or as in the rumen fluid 4:1:1:1. Negative controls consisted of charcoal-filtered air at 0.5L.min^{-1} passing through a 1L gas-wash bottle with a filter paper treated with 100µL of pure DCM or an empty 1L gas-wash bottle. 50 flies were tested for each treatment.

For experiments with *G. brevipalpis*, charcoal-filtered air at 5mL.min^{-1} passed through a 1L gas-wash bottle containing nothing or a polyethylene sachet (4×5.5cm, 1.5µm thickness) loaded with 1mL of carboxylic acid, and 500mL.min^{-1} passed through a 2nd gas-wash bottle filled with 5mL of pure acetone (99.8% pure, Acros). The two streams were mixed and introduced via an upright aluminium tube (7mm i.d.) through the tunnel floor at 25cm from the upwind end of the tunnel. This aluminium tube was bent downwind at a height of 40cm from the tunnel floor and entered a plume generator This consisted of a stainless steel cylinder (10.5cm long, 11cm o.d.) with an aluminium tapered end (4cm long, 11cm to 14cm o.d.) ending in by a steel grid (1mm mesh). A stimulus-controller (CS-55, Syntech, NL) switched the charcoal-filtered airflow between test and control flows for the successive 1min control and test periods. 40 flies (20 females + 20 males) were tested for each treatment.

The plume structure was visualised by generating a plume of ammonium acetate. Because of the plume generator and the semi-laminar flow screens, the odour plume could be described as a cone of ~10cm in diameter at 30cm from the upwind end of the wind tunnel and ~50cm in diameter at 5cm from the downwind end.

To avoid disturbance between flies that could have the effect of masking any activation of the flies by an odour, they were tested individually in the wind tunnel (Warnes 1989). Each fly was transferred into a plastic release cage (transparent PVC cylinders 15cm×10cm) with both ends covered with cotton netting (1mm mesh). The release cage was placed horizontally at the downwind end of the tunnel and after 1min of acclimatisation both doors were lifted slowly. If the fly did not exit during cage opening it was successively exposed for 1 min to odour-free air (negative control) and then for 1 min to the test product. Effects of test stimuli were quantified by 3 or 4 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 50cm within the plume of odour, *attraction* when the fly made a directed flight to within 10cm of the source. Differences in behavioural criteria between treatments were calculated using Pearson's Chi-squared test with Yates' continuity correction in R software.

For wind tunnel experiments with *G. brevipalpis* 1mL of pure synthetic carboxylic acid (Tab. 1) was put inside a polyethylene sachet (4×5.5cm 1.5µm thickness) before thermo-sealing. The release rate was then calculated after measuring the weight loss more than 20 times over 4 days (AE100 balance, Mettler-Toledo, Switzerland ; Annex G). The amount of compound released into the wind tunnel during the 1min experiment was then calculated as the Release Rate divided by 60. It permits an estimation of the flow of compounds [mg/L] inside the odour plume with the formula: $\frac{\text{Release Rate}}{60 \times \text{Volume}_{\text{plume}}}$ with $\text{Volume}_{\text{plume}}$ estimated to 120L/min.

Results

Rumen is a bioreactor containing a rich variety of partially oxidised products. With our fractionation method, we were able to isolate a phenolic and an acidic fraction with ease, and other fractions less easily.

Amines and neutral compound fraction

The yield in the amine and neutral fraction was low with our fractionation method, even when the first step in the extraction was repeated twice. In order to increase the amount of solute on the gas chromatography column used in the analysis of these fractions, a long retention gap and a large volume injection techniques was applied (Annex E). However, we were not able to satisfactorily characterise the constituents of this fraction as it depends on the plants fed on by the donor animal. But in other analyses made on the entire extract of rumen fluid (Syed, 2002; Jeanbourquin, 2005), different families of compounds were identified in this fraction: ketones (such as 6-methyl-5-hepten-2-one, octan-3-one, undecan-2-one), terpenes (such as β-cyclocitral, D-limonene, β-caryophyllene and α-humulene), aldehydes (from heptanal to dodecanal), sulphur compounds and aliphatic hydrocarbons. None of these molecules is exclusive to the rumen.

Phenolic fraction

4-methyl phenol (*p*-cresol) and 3-methyl indole (skatole) predominated in 4 different phenolic fractions extracted from different rumen fluid samples (Fig. 2). Only the *p*-cresol elicited a response from the antennal receptor cells of *G. pallidipes* and *G. brevipalpis*. Other phenols present at small amounts in this fraction did not elicit any EAG responses from the two species tested.

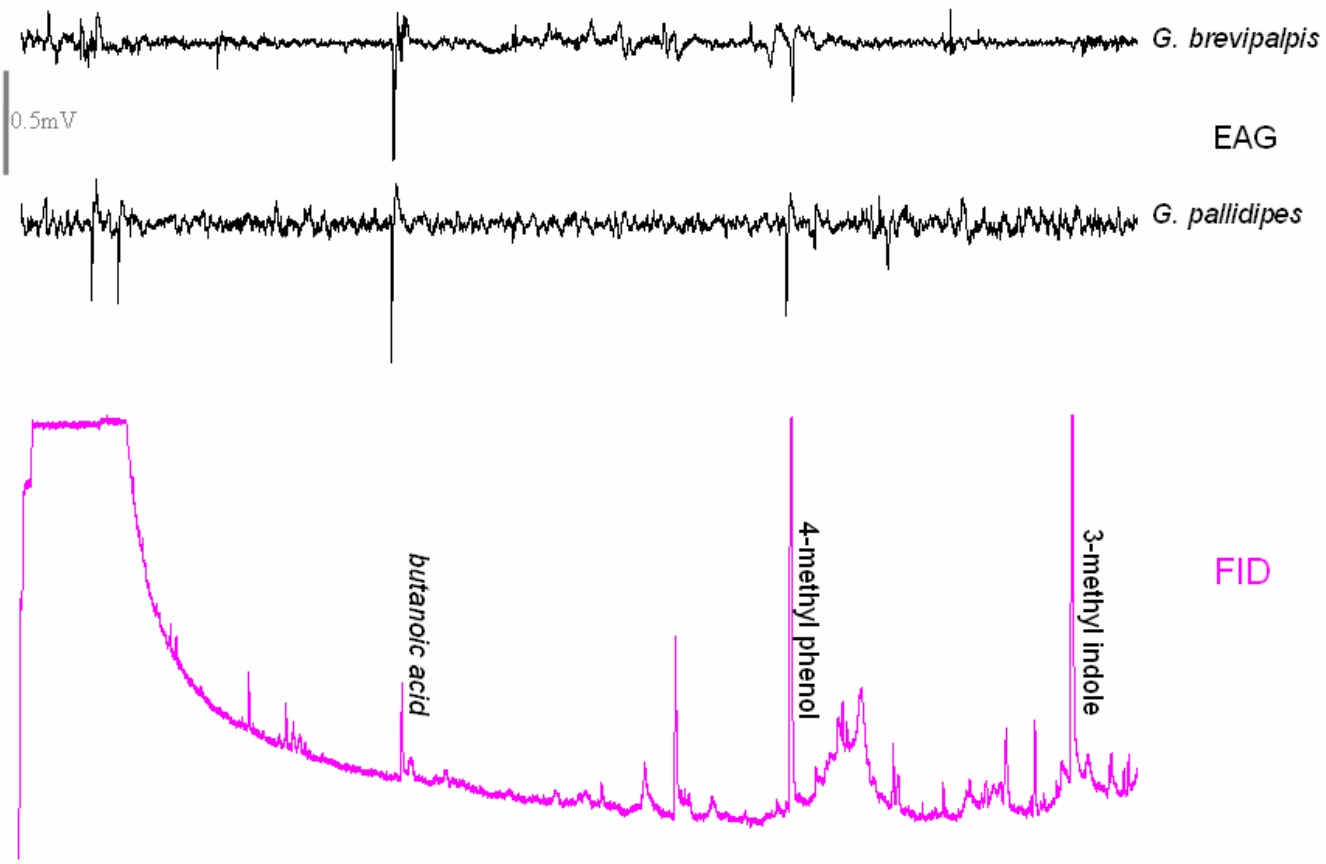

Figure 2. Responses of *G. pallidipes* and *G. brevipalpis* antennal olfactory cells to the phenolic fraction of rumen fluid as analysed by gas chromatography-coupled electrophysiological recordings.

The lower trace is the flame ionisation detector (FID) response of the chromatograph and the upper traces are the EAG responses of the antennal olfactory cells generated during elution of the biologically active constituents from the gas chromatographic column.
Scale bar in mV (common between *G. pallidipes* and *G. brevipalpis*) provides a measure of the depolarisations recorded.

Acidic fraction

Propanoic to hexanoic carboxylic acids and their branched isomers, i.e. isobutanoic and isovaleric acids are the major volatile constituents of rumen bolus. The relative composition and quantity of carboxylic acids remained almost identical in the 6 analyses (5µL-10µL) of our acidic fractions from 3 separate rumen fluid collections (Tab. 2). Butanoic acid was always the preponderant carboxylic acid and propanoic, isovaleric, valeric and hexanoic acids were present at amounts some 25% of the major constituent. All the other carboxylic acids were present at low amounts compared to butanoic acid, except benzenepropanoic acid for which the quantity was the most inconstant. This demonstrates that carboxylic acids are predictable end products of rumen metabolism, recurrently produced at similar levels. Variability in the amount of the aromatic carboxylic acids was much higher.

Table 2. The proportions of different carboxylic acids identified in 6 analyses of the acidic fraction from 3 separate rumen fluid collections, normalised with reference to the predominant component, butanoic acid.

	Mean quantity relative to butanoic acid	Standard deviation
propanoic acid	26.69	0.75
isobutanoic acid	6.62	0.63
butanoic acid	100	
isovaleric acid	28.10	3.30
valeric acid	28.85	2.27
hexanoic acid	26.87	6.50
heptanoic acid	1.27	0.69
cyclo hexane carboxylic acid	1.63	0.10
benzoic acid	*1.40*	*0.35*
benzenacetic acid	*4.10*	*0.85*
benzenepropanoic acid	*93.83*	*30.85*

The EAG responses of 3 tsetse species belonging to different subgenera responded in a remarkably similar manner to the carboxylic acids isolated in the acidic fraction of rumen fluid (Fig. 3). The aromatic carboxylic acids, benzoic, benzeneacetic and benzenepropanoic acids did not elicit any EAG response whereas the unbranched carboxylic acids butanoic, isovaleric, valeric and hexanoic acids evoked a high response for all 3 species. As the depolarisation of the antennal receptor cells is linked to the dose of a chemostimulant, the highest EAG responses in this fraction were elicited by the predominating unbranched components. The EAG response to cyclo hexane carboxylic acid was much stronger compared the amounts present in the fraction.

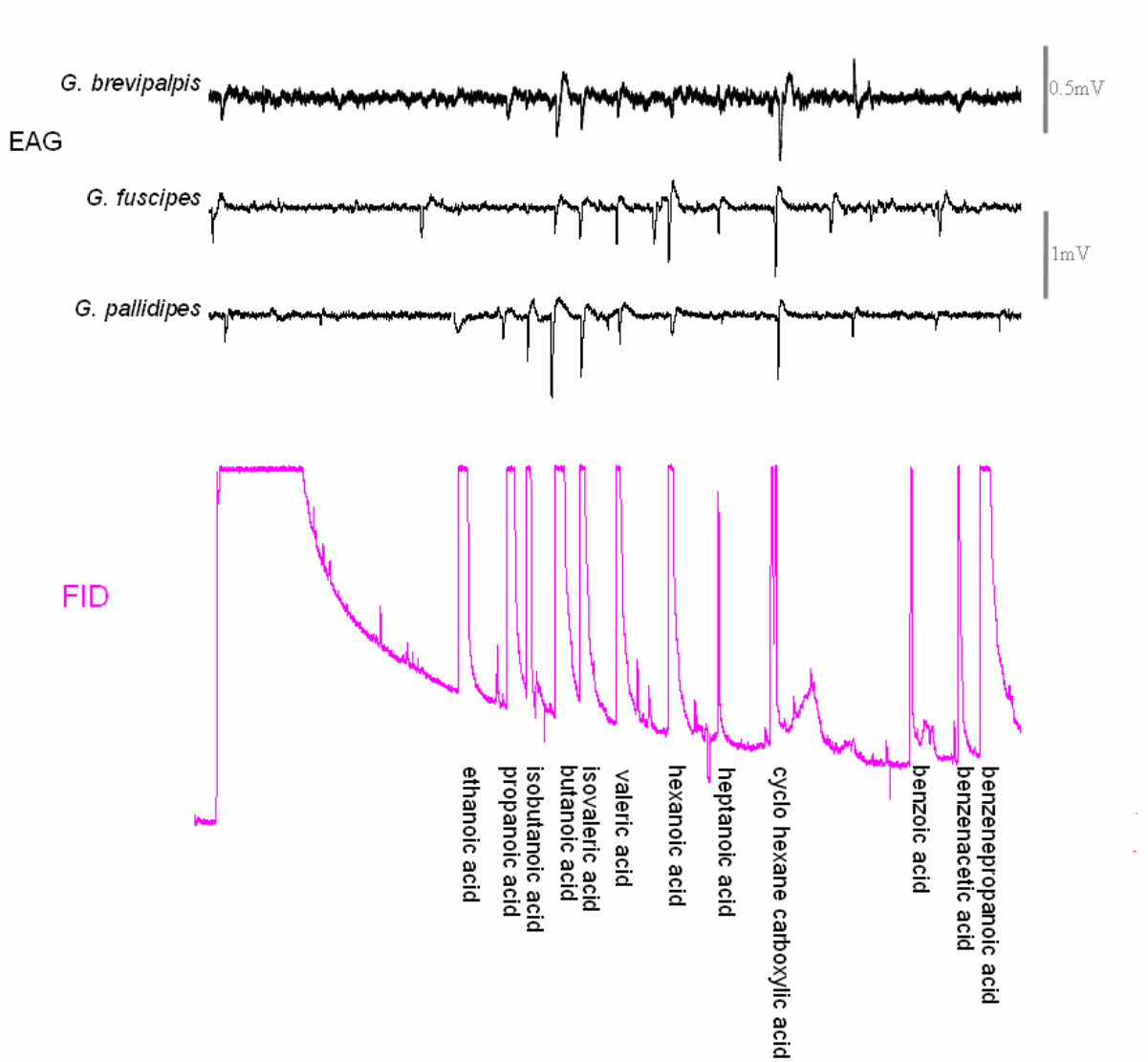

Figure 3. Responses of antennal olfactory cells of three tsetse fly species from different African habitats to the acidic fraction of rumen fluid.

The lower trace is the flame ionisation detector (FID) response of the chromatograph and the upper traces are the electrophysiological responses of the antennal receptor cells, the electroantennogram (EAG), generated during elution of the biologically active constituents of the extract from the gas chromatographic column (30m BGB-FFAP phase).
Scale bars in mV (common between *G. fuscipes* and *G. pallidipes*) provide a measure of the depolarisations recorded.

By analysing the EAG responses of *G. brevipalpis* to the different amounts of carboxylic acids present in the acidic fraction of rumen fluid and to known amounts of these products, we were able to estimate the threshold of the olfactory receptor cells for each of them (Fig. 4). Three different groups of carboxylic acids emerge from this analysis with a low threshold for cyclo hexane carboxylic acid, medium thresholds for ethanoic, isobutanoic and heptanoic acid, and the high thresholds for the most predominant carboxylic acids of the rumen, namely, propanoic, butanoic, isovaleric, valeric and hexanoic acids. This confirmed cyclo hexane carboxylic acid as the most active carboxylic acid constituent of the acid fraction of rumen fluid.

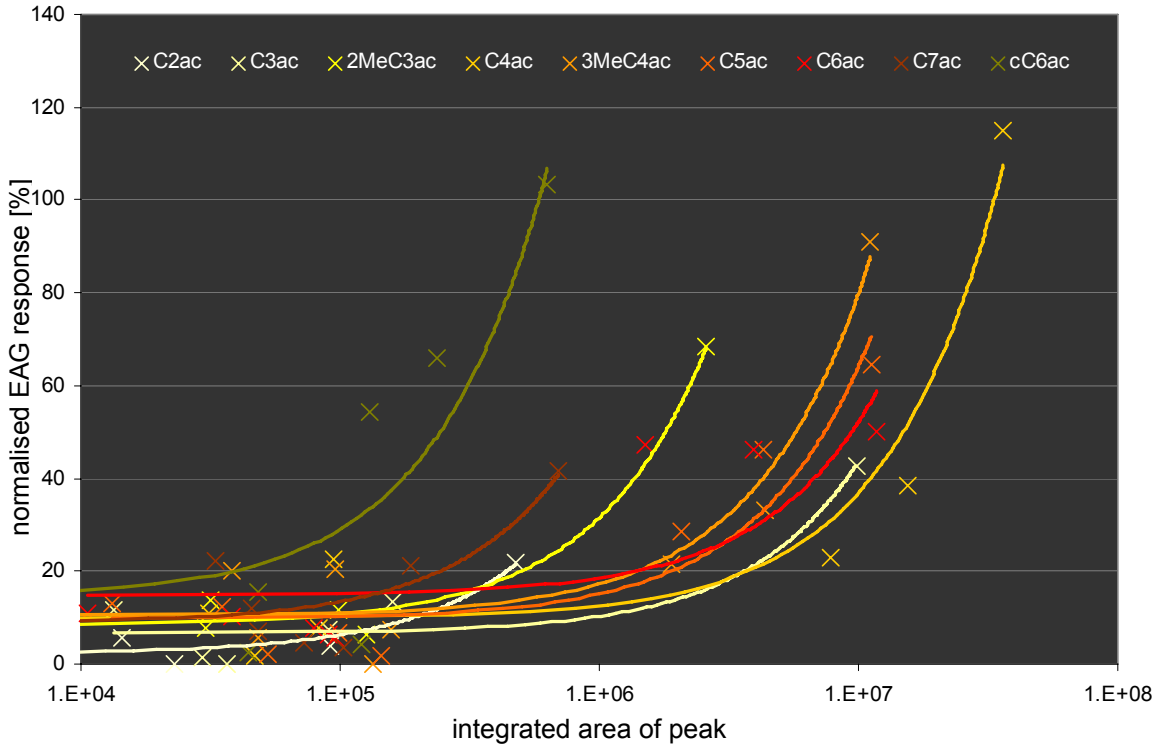

Figure 4. EAG responses of *G. brevipalpis* to different doses of volatile carboxylic acids present in the acidic fraction of rumen fluid and to standards.

The antenna responses were recorded during elution from the gas chromatographic column of the constituents of the carboxylic acid fraction of rumen fluid: ethanoic acid (*C2ac*, n=5 antennae), propanoic acid (*C3ac*, n=6), isobutanoic acid (*2MeC3ac*, n=6), butanoic acid (*C4ac*, n=8), isovaleric acid (*3MeC4ac*, n=9), valeric acid (*C5ac*, n=8), hexanoic acid (*C6ac*, n=8), heptanoic acid (*C7ac*, n=8) and cyclo hexane carboxylic acid (*cC6ac*, n=7).
The quantity (*integrated area of peak*) of products detected by the flame ionisation detector was calculated using an integrator (SP4270, Spectra Physics, USA). The antennal responses were normalised using a puff of air over 1µg of 1-octen-3-ol in a stimulus syringe as reference at the start and end of each analysis.

Behaviour responses of *G. pallidipes* were recorded in a wind tunnel by stimulating the flies with a mixture of the predominant carboxylic acids of the rumen fluid that elicited EAG response from the 3 tsetse fly species, namely butanoic, isovaleric, valeric and hexanoic acids (Fig. 3). An equimolar mixture of these 4 acids produced a significant increase of activation, but failed to elicit a significant increase in cage exit with only 9 flies activated and 4 flying from the cage over 50 flies tested (Fig. 5). However, a mixture of these products at a ratio similar to that present in the rumen (Tab. 2) induced significantly more activation and exit from the release cage. Of the 50 flies tested with this mixture 14 were activated and 5 reached the odour source. However, even though the 4 acid rumen-like blend elicited a stronger behavioural reaction it was not significantly different from that induced by the equimolar carboxylic acid mixture (Fig. 5). Moreover, both mixtures of these four carboxylic acids were prepared with DCM as solvent, this solvent affected the behaviour of *G. pallidipes* on its own with 5 of the 50 tested flies flying from the cage and 1 reaching the source such as no significant difference was recorded between tests with DCM alone and the two carboxylic acid mixtures for any of the 3 behavioural criteria recorded.

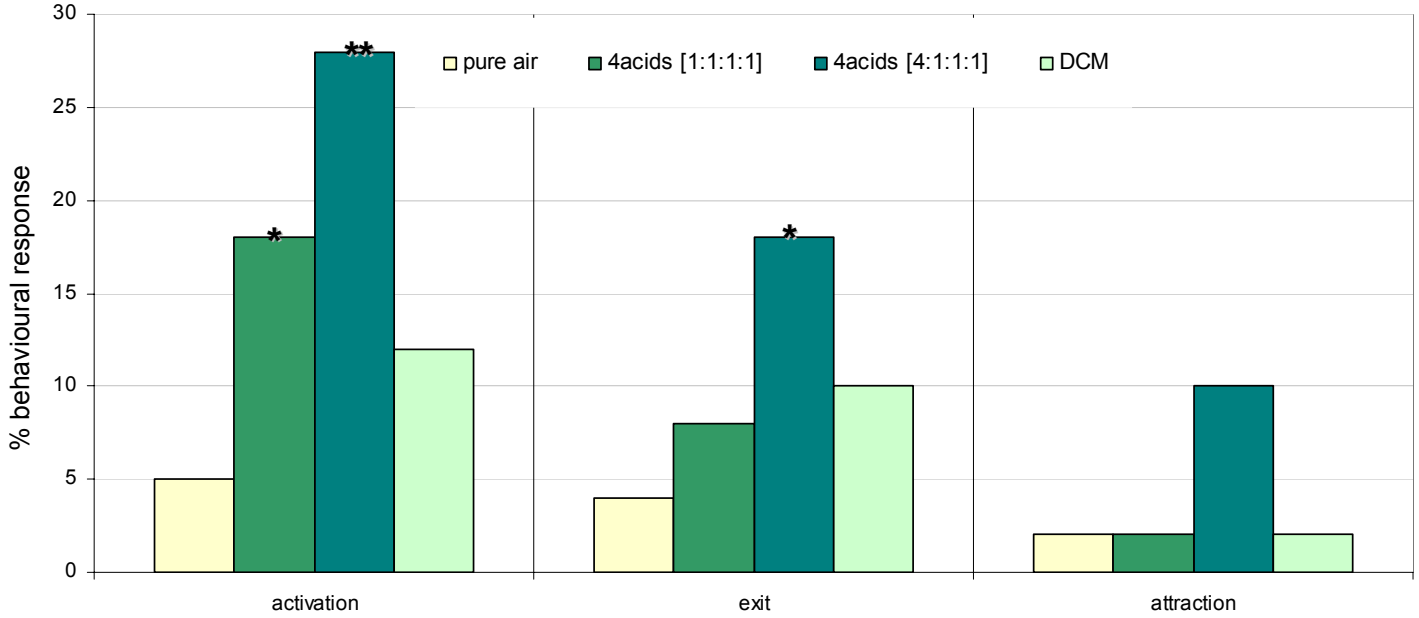

Figure 5. Behavioural responses of *G. pallidipes* to mixtures of 4 carboxylic acids mixed at different proportions and to the solvent alone.

The behavioural criteria recorded were: *activation*, the fly moved in the cage; *exit*, the fly flew from the cage; *attraction*, the fly made a directed flight within the plume of odour to within 10cm of the source. During the 1min test period, 0.5L of air passed through a 1L gas-wash bottle into which 100µL of the tested compounds were applied on a filter paper disk (diameter 9cm); *4 acids* is a mixture of butanoic, pentanoic, hexanoic and isovaleric acids, diluted at 100ng/µL in dichloromethane, with the respective ratios in brackets (equimolar 1:1:1:1 or as in rumen fluid 4:1:1:1). Dichloromethane (*DCM*) used for carboxylic acid mixture dilutions. *Pure air* (negative control) was charcoal-filtered air passing through an empty 1L gas-wash bottle. 50 flies were tested for each treatment. Asterisks indicate that the percentage response is significantly different from that of pure air at $P < 0.05$ (*) or 0.01 (**) levels of probability using Pearson's Chi-squared test with Yates' continuity correction.

In order to avoid the use of DCM, wind tunnel experiments with *G. brevipalpis* were made with carboxylic acids presented in a polyethylene sachet, as used in the field for tsetse flies with known attractants. The evaporation rates of the acids through the polyethylene were different, depending on the structures of the molecules (Tab. 3).

Table 3. Release rate of acetone present neat in a gas-wash bottle and different carboxylic acids as dispensed from a polyethylene sachet.

	acetone	isobutanoic acid	butanoic acid	isovaleric acid	valeric acid	hexanoic acid	cyclo hexane carboxylic acid
release rate [mg/h]	19.2	1.9	3.7	1.4	2.7	2.1	0.4
mean quantity injected into the odour plume [µg/L]	2.67	0.26	0.51	0.19	0.38	0.29	0.06

As in the wind tunnel experiments, a polyethylene sachet (4cm×5.5cm, film thickness 150µm) was filled and sealed with 1mL of a carboxylic acid. A mean evaporation rate was calculated by weighings over 4 consecutive days (n>20) while hanging in a fume hood with a draught (900m^3/h); 5mL of acetone were deposited neat inside a 1L gas-wash bottle.
An approximation of the quantity delivered in the odour plume was calculated (see Material & Methods for details).

Contrary to *G. pallidipes*, *G. brevipalpis* showed no spontaneous activity to the negative control (pure air). Acetone alone changed significantly the 4 behavioural criteria, with 8 flies reaching the source of the 40 flies tested (Fig. 6). The carboxylic acids tested were isobutanoic, butanoic, isovaleric, pentanoic, hexanoic and cyclo hexane carboxylic acids. Presented alone, these acids did not induce any behaviour responses, but by adding anyone of these carboxylic acid to acetone activation and release cage exit were significantly higher with all the mixtures tested. Between 11 and 19 flies were activated by the mixtures of acetone plus carboxylic acids tested, but only 3 to 6 reached the source. However, four of these mixtures, acetone added to isobutanoic, valeric, hexanoic, cyclo hexane carboxylic acids, were significantly better than the negative control in eliciting directed flight to the source. Moreover, cyclo hexane carboxylic acid plus acetone always induced a higher behavioural response than acetone alone, with 12 of the 19 activated flies making a directed flight as against only 9 with acetone on its own. For all fly behaviours recorded there were no significant increases or decreases in the responses induced by stimulation with acetone alone compared to acetone plus anyone of the carboxylic acids.

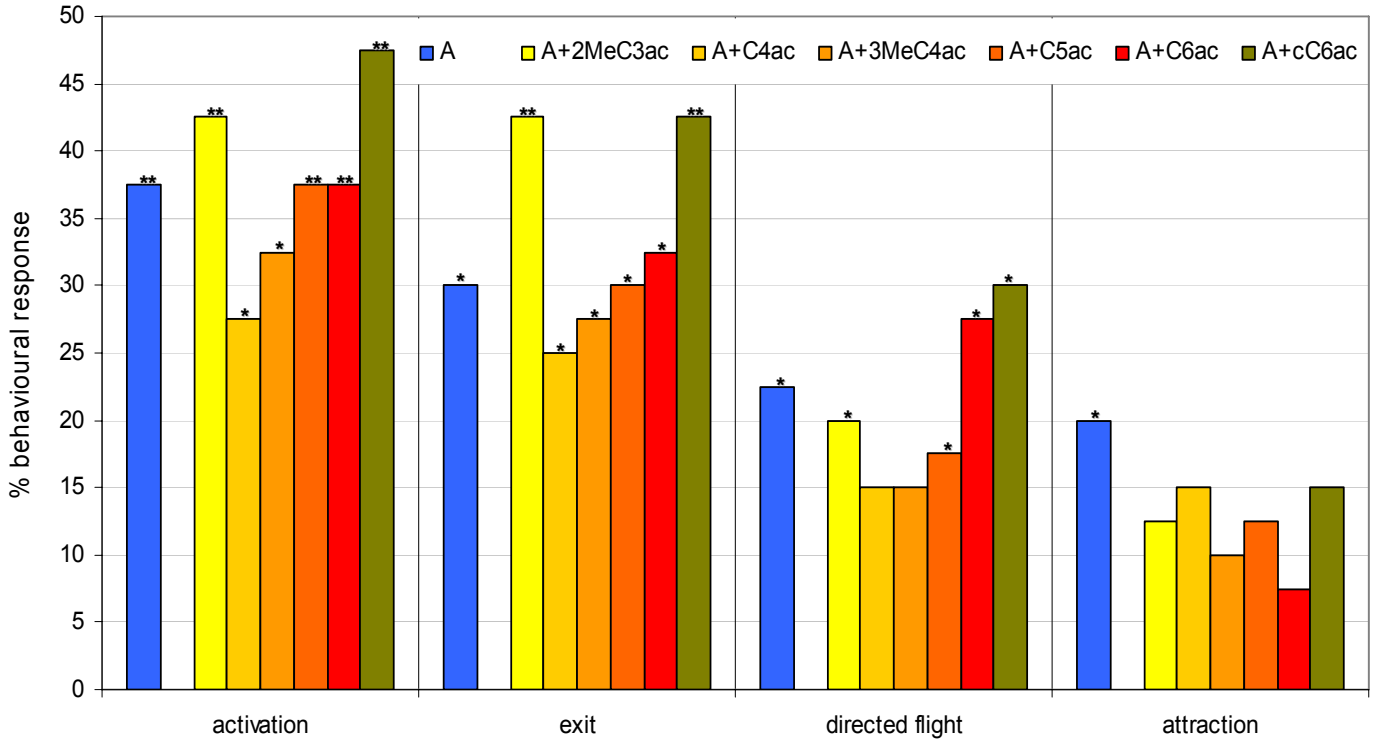

Figure 6. Behavioural responses of *Glossina brevipalpis* to acetone and mixtures of acetone plus one of the carboxylic acids found in the rumen.

Behavioural criteria recorded were as described in the legend to *Figure 5*.
During the 1min test period, 0.5L of pure air passing through a 1L gas-wash bottle with 5mL of acetone (*A*) was mixed with 5mL of pure air passing through a second 1L gas-wash bottle in which a polyethylene sachet (4cm×5.5cm, film thickness 150µm) containing 1mL of either isobutanoic acid (*2MeC3ac*, released at 1.9mg/h), butanoic acid (*C4ac*, 3.7mg/h), isovaleric acid (*3MeC4ac*, 1.4mg/h), valeric acid (*C5ac*, 2.7mg/h), hexanoic acid (*C6ac*, 2.1mg/h) or cyclo hexane carboxylic acid (*cC6ac*, 0.4mg/h). During negative controls with charcoal-filtered air passing at 0.5L/min no behaviour responses were recorded.
40 flies were tested for each treatment; asterisks indicate that the percentage response is significantly different from that of pure air at $P < 0.05$ (*), 0.01(**) or 0.001 (***) levels of probability using Pearson's Chi-squared test with Yates' continuity correction.

Discussion

Previous studies already demonstrated the occurrence of phenolic compounds in rumen (Donzé, McMahon & Guerin, 2004; Jeanbourquin & Guerin, 2007; Syed, 2002) where their presence is attributed to the degradation by rumen bacteria of 2 amino acids: tyrosine forms phenols and various alkyl phenols, and tryptophan is transformed into indole and skatole. Results presented here confirm their presence as rumen constituents and consistent EAG responses were recorded to *p*-cresol in *G. pallidipes* and *G. brevipalpis*. Phenols are the key volatile components of cattle urine that attract tsetse (Owaga, 1985; Bursell *et al.*, 1988; Vale *et al.*, 1988), and *p*-cresol is one of the electrophysiologically active phenols that in mixture induced upwind flight in wind tunnel tests and increased trap catches of *G. m. morsitans* and *G. pallidipes* in field trials in Zimbabwe (Bursell, Gough et al. 1988). Nowaday, *p*-cresol is used with 1-octen-3-ol, acetone and an other phenol isolated from cow urine 3*n*-propyl phenol to attract tsestse flies to visual traps (Vale, Hall et al. 1988). 3*n*-Propyl phenol was not detected in our phenolic fraction of rumen fluid as in urine, only a small amount of 3*n*-propyl phenol is formed in the rumen (Hassanali *et al.* 1986) and because the amount of phenol in our fraction was relatively low, we can suggest that minor phenolic components were lost during chemical fraction.

Carboxylic acids are the major compounds present in the rumen (Clarke and Bauchop 1977; Mackie, Stroot et al. 1998). They are not specific of the bioreactor, and may also be released from protected areas of the host pelage where oxygen may be limiting such as the axillar and genital areas (Höller, Breves et al. 1989; Wood 1997). In the rumen they are produced in large quantity through the degradation by microbes of plant carbohydrates and aliphatic amino acids.

In our acidic fractions of rumen fluid, 12 different carboxylic acids were recovered in reproducible amounts. Contrary to the aromatic carboxylic acids, the EAG responses of the 3 tsetse species of different subgenera responded in a remarkably similar manner to all the other carboxylic acids, suggesting that sensibility to these compounds has been conserved through evolutionary history in this group of haematophagous insects. The antennal receptor cell threshold of *G. brevipalpis* for these compounds is inversely related to the quantity in rumen fluid. Whereas heptanoic and cyclo hexane carboxylic acids are present at low amounts in the rumen, the antenna threshold for these products is very low, and the opposite for propanoic, butanoic, isovaleric, valeric and hexanoic acids present at high amounts in the rumen fluid. However compared to other compounds used as attractants like 1-octen-3-ol or *p*-cresol, even the lowest threshold of all these compounds is relatively high (annex F).

A mixture of the 4 predominant carboxylic acids at the ratio prevalent in rumen fluid of 4:1:1:1, butanoic:isovaleric:valeric:hexanoic acids elicited significant activation and cage exit by *G. pallidipes* in the wind tunnel. The ratio of these carboxylic acids seems to be important as the ratio equivalent to that of rumen fluid increased more the behaviour responses of *G. pallidipes* the 3 criteria measured than an equimolar mixture of these acids. However, the solvent used to dilute these carboxylic acids, dichloromethane (DCM), played a role in the behavioural responses recorded. Attraction to DCM was already recorded by Bernier *et al.* (2003) for *Aedes aegypti* and by Jewett & Bjostad (1996) for diabroticite larvae. The latter authors explain this attraction by a structural similarity between the DCM molecule and CO_2. With this hypothesis, the behavioural responses of *G. pallidipes* to DCM could be explained by the activation of CO_2 receptors by the DCM molecule. CO_2 is a strong attractant for *Glossina* spp. (Rennison and Robertson 1960; Torr 1990) which could influence fly stimulation induced by the mixture of carboxylic acids.

The addition of different carboxylic acids to acetone did not increase or decrease significantly the effect of acetone on the behaviours of *G. brevipalpis* recorded. Whereas attraction to acetone was significantly higher than to the control, this was not the case when anyone carboxylic acid was added to acetone. The carboxylic acids showing the lowest sensory threshold, i.e. isobutanoic acid and cyclo hexane carboxylic acid showed better behavioural effects, with acetone plus cyclo hexane carboxylic acid evoking higher behavioural effects than acetone under 3 of the 4 behaviours criteria even though these differences were not significant.

Carboxylic acids and oxycarboxylic acids have been implicated in the chemical ecology of haematophagous arthropods. Olfactory receptor cells of haematophagous arthropods responding to straight chain and branched carboxylic acids have already been reported and their importance for host location has been widely demonstrated in mosquitoes (Bowen, 1995; Cork, 1996; Knols *et al.*, 1997; Meijerink & Van Loon, 1999; Constantini *et al.*, 2001), sandflies (Dougherty et al., 1999), ticks (Donzé et al., 2004; McMahon, 1999; Steullet & Guerin, 1994) and triatomine bugs (Guerenstein & Guerin, 2001), but carboxylic acids had not yet been tested extensively on tsetse. Hughes (1957) working with *G. palpalis* and *G. morsitans* found that iso-valeric and butanoic acid induced flight activity linked to product dose. Moreover, he recorded an increase probing responses of these 2 species in the presence of propanoic, butanoic or valeric acid vapours. Warnes (1990) tested the addition of fractions of sebum to traps in attracting *G. m. morsitans* and *G. pallidipes*. The acidic fraction did not elicit an increase in attraction nor a repellent effect on both species. More specific tests with the addition of synthetic carboxylic acids have been done in the field by Vale (1980) and by Torr *et al.* (1996), but these tests were made with very high doses of C_1 to C_8 acids, 2 to 150 times higher than used here, which could explain the poor level of attraction obtained (Tab. 4). The carboxylic acids tested were added to known

Table 4. Field efficacy of the addition of some carboxylic acids to visual traps.

compounds	dose [*mg/h*]	added to	results	ref
propanoic ac.	400	CO_2 + acetone	non significant increase	a
butanoic ac.	5	POCA	non significant decrease	b
	20	POCA	non significant decrease	b
	300	CO_2 + acetone	non significant increase	a
pentanoic ac.	10	*nothing*	non significant decrease	b
	10	POCA	significant decrease (P<0.1)	b
	100	CO_2 + acetone	non significant decrease	a
hexanoic ac.	5	POCA	significant decrease (P<0.05)	b
	100	CO_2 + acetone	significant decrease (P<0.001)	a
isobutanoic ac.	300	CO_2 + acetone	non significant decrease	a
isovaleric ac.	5	POCA	non significant decrease	b
	100	CO_2 + acetone	non significant decrease	a

The doses of the compounds added were: *CO_2* (2.5L/min) *+ acetone* (3000mg/h) and *POCA*, a blend of 3*n*-propyl phenol (0.1mg/h) plus 1-octen-3-ol (0.4mg/h) plus *p*-cresol (0.8mg/h) plus acetone (500mg/h). Letters refer to the works of ***a***: Vale (1980) and ***b***: Torr *et al.* (1996).

attractants (CO_2 plus acetone or *3n*-propyl phenol, 1-octen-3-ol, *p*-cresol plus acetone) but at doses 6 to 100 times higher. It is well known that high concentrations of carboxylic acid act as repellents and can decrease the efficacy of approaches in mixtures (Knols, Van Loon et al. 1997). Traps with isovaleric acid dispensed alone at 1.25µg/h did not catch significantly more flies than traps not baited, but added to 3*n*-propyl phenol, 1-octen-3-ol, *p*-cresol and acetone this product contributed to an increase in trap capture of *G. austeni*, *G. pallidipes* and *G. brevipalpis* (IAEA-TECDOC 2003). In our experiments, the amount of compounds tested is in the range of what is released by a cow: acetone 5mg/h and 1-octen-3-ol 43µg/h (Hall et al., 1984; Paynter & Brady, 1993). Moreover, compounds are usually released in a larger amount from the traps in the field: acetone at 150mg/h, 1-octen-3-ol at 0.5mg/h and *p*-cresol at 1.5mg/h (FAO 1992). Perhaps the amount of carboxylic acids released in the vapour phase was not high enough to influence the behaviour of *G. brevipalpis* already induced by acetone. On the other hand, cyclo hexane carboxylic acid which showed the lowest sensory threshold of the 9 carboxylic acids tested using the EAG method, induced the biggest changes in the behaviours of *G. brevipalpis* even though this acid had the lowest release rate of the 6 carboxylic acids tested.

The rumen of herbivores contains a large variety of volatile compounds regularly eructated in the atmosphere. Among them is *p*-cresol which is a known attractant for tsetse flies and a large quantity of carboxylic acids not extensively tested on tsetse flies to date. Most of these carboxylic acids are perceived by the three *Glossina* spp. tested here, but at relatively high thresholds compared to other compounds already used as attractant.

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Chapter 3

Metabolites present in breath influence the sensory ecology of tsetse flies, *Glossina* spp.

Abstract

The attractiveness of human breath as tested in the wind tunnel for *G. brevipalpis* was found to be affected by its dilution. This dilution was reflected in the CO_2 content of the test treatment, but CO_2 failed to elicit significant behavioural responses when employed alone. This suggested to search for other products in breath that can affect the olfactory sensory system of tsetse fly spp. to compounds from various chemical families commonly found in human and cattle breath. Among C_6 to C_{11} alyphatic aldehydes, the lowest antenna threshold of *G. pallidipes* was recorded to nonanal, decanal and undecanal. Among terpens stereoisomers of caryophyllene elicited the strongest antennogram response from *G. pallidipes*. Among a set of ketones tested, best antennogram responses were obtained for 3-octen-2-one which can be formed during grass mastication. Tsetse flies were able to detect sulphid compounds such as dimethyl trisulphide and benzothiazole. The importance of products from these different chemical families present in breath is discussed in relation to the biosynthetic pathways from which they arise.

Key words : tsetse flies, olfaction, breath, electroantennogram recording, wind-tunnel, CO_2, aldehydes, terpens, ketones, sulphides.

Introduction

Breath emitted by a host is, on its own, a well known attractant for haematophagous arthropods such as ticks (McMahon and Guerin 2002), mosquitoes (Healy and Copland 1995) and biting flies (Warnes & Finlayson, 1985a; Warnes, 1990b). Even though breath is a complex mixture of several gases and more than a hundred volatiles, it is its CO_2 content that plays a major role as activator for almost all the blood-sucking insects (Rennison & Robertson, 1960; Caresta & Horner, 1968; Roberts, 1972; Taneja & Guerin, 1995; Constantini *et al.*, 1996; Cork, 1996; McMahon & Guerin, 2002; Barrozo & Lazzari, 2006). However breath-related chemicals are perceived by haematophagous arthropods and increase the attractive effect of CO_2. An example is acetone, which is found in breath and is one of the major tsetse fly attractants (Vale 1980).

Human breath has been widely investigated as it can be used as a medical tool for non-invasive diagnostics (Krotoszynski *et al.*, 1977; Wahl *et al.*, 1996; Phillips *et al.*, 1999). Similar studies with the same aim have been made on cattle (Elliott-Martin *et al.*, 1997; Spinhirne *et al.*, 2004). Even though there are some differences between individuals in both the case of humans and cattle, dependent of their physiological state, breath extracts share a common profile across vertebrates. For this reason, breath components for man and cattle have been compared in this study (Tab. 1) and used to choose breath components to be tested as chemostimuli for chemosensory cells on tsetse fly antennae. Having shown that human breath provides a reliable attractant for tsetse flies in the wind tunnel, the responses of these flies were then recorded to key breath components using the electroantennogram techniques.

Previously, behavioural tests in a wind tunnel have been done to confirm the attractiveness of human breath to *G. brevipalpis*. Two different breath dispensers were used to compare the power of stimulation of CO_2 which was tested alone at different concentrations.

Table 1. List of the most common organic compounds identified by different authors in human and/or cow breath.

chemical family	compound	human	cow	chemical family	compound	human	cow
alkanes	isoprene	ADEFGHJ		alcohols	methanol	ADH	
	pentane	FGHJ			ethanol	ADFG	
	trimethylpentane	FGH			propan-2-ol	AH	d
	hexane	AFG	d		pentanol	AFG	
	3-methylhexane	FGH			2-ethyl-1-hexanol	AG	c
	cyclohexene	GH		aromatics	phenol	G	d
	methyl cyclohexane	AFGH			p-cresol		c
	heptane	AFGHJ	d		2-ethyl phenol		c
	dimethyl heptane	AFH			styrene	HJ	d
	octane	AHJ	d		benzene	AGHJ	
	nonane	AH	d		ethylbenzene	AGHJ	
	decane	AHJ	d		xylene	AGJ	
	dodecane	AH			toluene	AGHJ	bd
terpenes	limonene	AGH		carboxylic acids	acetic acid	GH	bd
	camphor		c		propanoic acid	G	cd
	beta-caryophyllene		c		butanoic acid		c
ketones	acetone	ACDEGH	abd		isobutanoic acid		c
	acetophenone	G	d		pentanoic acid		c
	2,3-butanedione		d		isovaleric acid		cd
	butan-2-one		ab		hexanoic acid		cd
	methyl-4-heptanone	AH		sulphides	hydrogen sulphide	BI	
aldehydes	acetaldehyde	ACEG	d		methanethiol	BCGI	
	hexanal	G			dimethyl sulfide	ABCI	
	heptanal	G			carbon disulfide	EFI	
	octanal		d		dimethyl disulfide	I	d
	nonanal	H	c		benzothiazole	H	
	decanal	AH	cd				

References for human breath constituents **A**: Krotoszynski *et al.* 1977, **B**: Tonzetich *et al.* 1978, **C**: Manolis *et al.* 1983, **D**: Jones *et al.* 1985, **E**: Phillips *et al.* 1991, **F**: Phillips *et al.* 1992, **G**: Wahl *et al.* 1996, **H**: Phillips *et al.* 1999, **I**: Rodriguez *et al.* 2002, **J**: Poli *et al.* 2005.

References for cow breath constituents **a**: Torr *et al.* 1995, **b**: Eliott-Martin *et al.* 1997, **c**: Syed 2002, **d**: Spinhirne *et al.* 2004.

Materiel & Methods

Insects

Tsetse flies were held in environmental conditions similar to those described in the previous chapter.

Stimulations with human breath in the wind tunnel

The responses of the forest tsetse fly species *G. brevipalpis* to human breath were recorded in the wind tunnel. Human breath was delivered into the wind tunnel in three different manners. The simplest was achieved by blowing directly through a 5mm i.d. silicone tube into the wind tunnel linked to the aluminium tube and the plume generator (see below). However, this method of stimulation could not be well standardized. To permit the standardisation of the test with human breath, 5-10 minutes before the start of the morning experimental sessions, human breath was blown for 3min into 2 different delivery systems: a 1L gas-wash bottle and a Tedlar® bag (25L, SKC, USA). These breath sampling steps were made inside the climate chamber housing the wind tunnel in order to keep similar conditions of temperature and air composition for both filling and emptying of the Tedlar® bag and gas-wash bottle. Breath from the 1L gas-wash bottle was evacuated by flushing with charcoal-filtered air at 0.5L/min into the wind tunnel via a 2mm i.d. teflon tube linked to the aluminium tube and the plume generator (see below). Thereby, the 1L content of the gas-wash bottle was diluted by a factor of two in successive stimulations with the same breath volume. Warnes (1990) and Green (1993) already used the Tedlar® gas sampling bag to collect, store and test ox breath on tsetse flies. In our case, breath was sucked from the Tedlar® bag by using a pump (MPC 100-E, Labovac®, Saskia, Germany) and released into the wind tunnel via a 2mm i.d. teflon tube linked to the aluminium tube and the plume generator (see below). Other behavioural tests were made with *G. brevipalpis* exposed to CO_2 increases of 80ppm, 387ppm and 708ppm above background with CO_2 from a gas tank containing 2% of CO_2 in air and released into the wind tunnel by connecting the CO_2 gas tank outlet with a 1mm i.d. stainless steel tube linked to the aluminium tube and the plume generator (see below). Negative control consisted to charcoal-filtered air dispensed at 0.5L/min.

All the air streams (tests and control) were introduced via an upright aluminium tube (7mm i.d.) passing through the tunnel floor at 25cm from the upwind end of the tunnel. This aluminium tube was bent downwind at a height of 40cm from the tunnel floor and entered a plume generator. This consisted of a stainless steel cylinder (10.5cm long, 11cm o.d.) with an aluminium tapered end (4cm long, 11cm to 14cm o.d.) closed off by a bronze grid (1mm mesh). A stimulus controller (CS-55, Syntech, NL) switched the charcoal-filtered airflow between test and control flows for the successive 1min control and test periods.

CO_2 levels (in ppm) were measured at 5cm distance from the stimulus outlet using an IR CO_2 analyzer (0-2000ppm range; Binos®1, Leybold-Heraeus, Germany) during evacuation of treatments from the gas-wash bottle, the Tedlar® bag and the CO_2 tank.

Behavioural experiments

The wind tunnel and the behavioural recording methods used for these experiments were similar to those described in the previous chapter.

Electroantennogram recordings

The methods used to record electroantennogram (EAG) responses and gas-chromatography linked electroantennogram detector (GC-EAD) responses to test stimuli were as described in the previous chapter. The compounds used for these recordings are listed in the table 2. They were diluted in dichloromethane (DCM, Merck, analytical grade) to obtain solutions at different amounts/µL.

Table 2. List of the compounds tested in the electroantennogram assays and by gas chromatography linked antennogram detector recordings.

name	compound class	purity	firm
hexanal	aldehydes	99%	Fluka
heptanal		97%	Fluka
octanal		>98%	Fluka
nonanal		97%	Fluka
decanal		97%	Fluka
undecanal		97%	Fluka
Mix with germacrene D	terpenes		Firmenich
β-caryophyllene		99%	Fluka
β-cyclo citral		96%	Fluka
3-octen-2-one	ketones	>98%	Aldrich
2-undecanone		>98%	Merck
6-methyl-5-hepten-2-one		98%	Fluka
dimethyl disulfide	sulphides	99%	Fluka
dimethyl trisulfide		98%	Sigma-Aldrich
benzothiazole		98%	Roth
1-octen-3-ol	alcohols	>97%	Merck

Results

When breath was blown directly into the wind tunnel 83% of the 246 flies tested were activated, 72% exited the release cage 51% reached the source. This large number of flies tested was spread on 52 days with a mean of 8.3 flies tested each day.

The responses of *G. brevipalpis* to more standardised human breath delivery were reported in figure 1. Three behavioural criteria recorded namely activation, cage exit and attraction, were significantly different from the negative control during the first 4 stimulations using the gas-wash bottle. During these 4 consecutive samples each requiring displacement of 0.5L from the bottle and equivalent to 2 fold emptying of the bottle, more than 60% of the flies were activated and a mean of 44% reached the source. The percentage of flies responding decreased

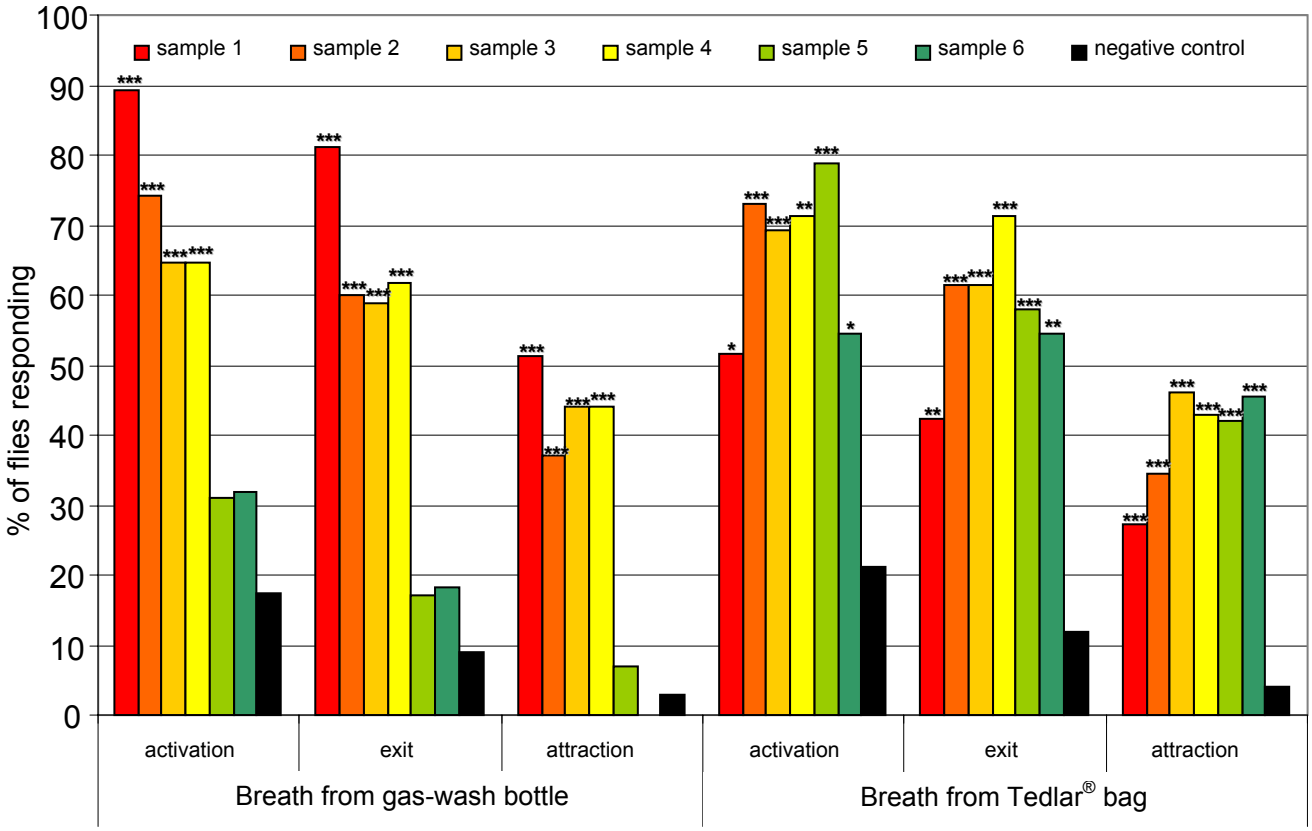

Figure 1. Behavioural responses of *G. brevipalpis* to 6 successive experiments with breath from a 1L gas-wash bottle and a Tedlar® bag filled with human breath.

The behavioural criteria recorded were: *activation*, the fly moved in the cage, *exit* the fly flew from the release cage; *attraction*, the fly made a directed flight to within 10cm of the source.
Breath was blown for 3min just before each experiment into the gas-wash bottle and the Tedlar® bag.
Asterisks indicate that the response rate is significantly different from the negative control (air alone) at P< 0.05 (*), 0.01 (**) or 0.001 (***) levels of probability using Pearson's Chi-squared test with Yates' continuity correction.

during subsequent dilution of the breath contained inside the gas-wash bottle and the 2 last stimulations from the bottle did not elicit any significant behaviour. Activation declined from 89% to 65% during the first 4 evacuations and then fell to 32% of activated flies in the 2 last stimulations with the same 1L gas-wash bottle. On the other hand, behavioural responses of *G. brevipalpis* to human breath sucked out from the Tedlar® bag were always significantly higher than air alone during 6 successive displacements of 0.5L from the 25L Tedlar® (Fig 1). But in its entirety, behavioural responses under the 4 criteria recorded were similar between successive evaluations of breath from the Tedlar® bag.

CO_2 concentration in the 2 breath delivery systems were measured (Fig 2) to show that the CO_2 level remained constant from the Tedlar® bag (253±10ppm) whereas it decreased from more than +400ppm to +30ppm during the stimulation with the gas-wash bottle.

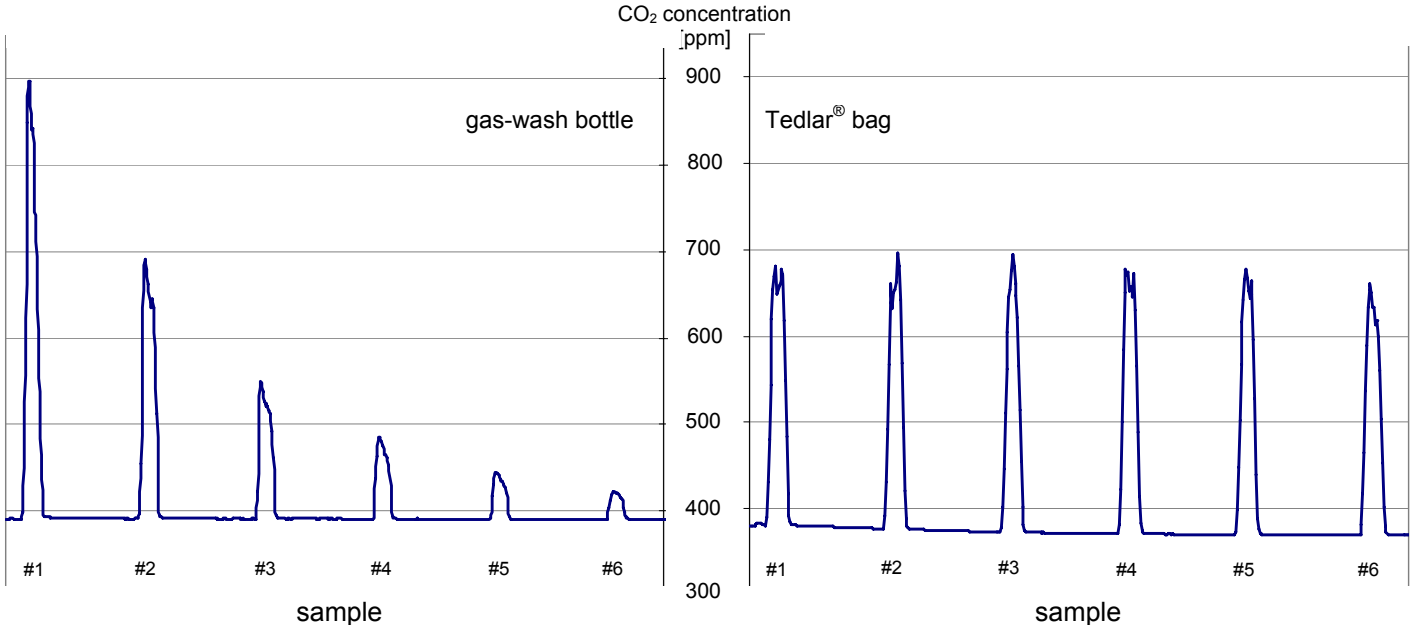

Figure 2. CO_2 levels measured at 5cm from the stimulus outlet in the plume of breath from a gas-wash bottle and a Tedlar® bag filled with breath at the beginning of the experiment.

Each delivery of breath lasted 1minute and was separated from the following passage for 7minutes of air alone. CO_2 levels in ppm were measured at 5cm distance from the stimulus outlet using an IR analyser (0-2000ppm range; Binos®1, Leybold-Heraeus, Germany) during evacuation from the gas-wash bottle and the Tedlar® bag.

Unlike constant responses of *G. brevipalpis* recorded to human breath evacuated from the Tedlar® bag, the significant difference between the fourth and the sixth evacuations from the gas-wash bottle seems to be linked to CO_2 decrease (Tab. 3). Tests made with pure CO_2 at 3 different concentrations failed to elicit any significant behaviour from *G. brevipalpis* in the wind-tunnel with only 9 of the 14 flies activated undertaking a flight and 3 arriving at the source of 92 tested among the 3 CO_2 concentrations.

Table 3. Behavioural responses of *G. brevipalpis* in 6 successive experiments to human breath from a 1L gas-wash bottle and a 25L Tedlar® bag filled with human breath, and to CO_2 at 3 different concentrations.

gas-wash bottle	activation [%]		exit [%]		attraction [%]		CO_2 increase
sample 1	89.2	a	81.1	a	51.4	a	376.7ppm
sample 2	74.3	ac	60.0	ac	37.1	a	242.1ppm
sample 3	64.7	ac	58.8	ac	44.1	a	127.8ppm
sample 4	64.7	ac	61.8	ac	44.1	a	74.2ppm
sample 5	31.0	bc	17.2	b	6.9	b	43.3ppm
sample 6	31.8	abc	18.2	bc	0.0	b	26.3ppm
negative control	17.5	b	9.1	b	2.9	b	0ppm
Tedlar® bag	activation [%]		exit [%]		attraction [%]		CO_2 increase
sample 1	51.5	a	42.4	a	27.3	a	265.9ppm
sample 2	73.1	a	61.5	a	34.6	a	256.7ppm
sample 3	69.2	a	61.5	a	46.2	a	245.0ppm
sample 4	71.4	a	71.4	a	42.9	a	261.6ppm
sample 5	78.9	a	57.9	a	42.1	a	251.1ppm
sample 6	54.5	a	54.5	a	45.5	a	238.7ppm
negative control	21.1	b	11.9	b	4.1	b	0ppm
CO_2	activation [%]		exit [%]		attraction [%]		CO_2 increase
3 levels	20.5	a	7.7	a	2.6	a	80ppm
	13.6	a	13.6	a	9.1	a	387ppm
	9.7	a	9.7	a	0.0	a	708ppm
negative control	7.1	a	2.7	a	2.7	a	0ppm

The behavioural criteria recorded were: *activation*, the fly moved in the cage, *exit* the fly flew from the release cage; *exit*, the fly flew from the release cage; *attraction*, the fly made a directed flight to within 10cm of the source.
negative control (n=274, 194 & 113) is charcoal-filtered air that induced no increase of CO_2 compared to ambient air. Breath was blown for 3min just before the experiment into the *gas-wash bottle* (n=37, 35, 34, 34, 29, 22) and the *Tedlar® bag* (n=33, 26, 26, 21, 19, 11). *pure CO_2* (n=39, 22, 31) was delivered from a gas tank containing 2% of CO_2 in air and injected into the wind tunnel via an aluminium tube (i.d. 5mm).
For more detail see *Figure 1* and *Material & Methods*

By plotting the percentage of flies responding under the different behavioural criteria to the amounts of CO_2 in human breath and alone from the gas tank, we conclude that there are more important compounds other than CO_2 in breath that elicit activation, cage exit and attraction of *G. brevipalpis* (Fig. 3). The behavioural responses to breath show a logarithmic increase dependent on the amount of CO_2 for each of the 3 behavioural criteria recorded, whereas stimulation with pure CO_2 alone from the gas tank elicited mainly activation, and this, in a manner independent of dose. Moreover, where breath from the Tedlar® bag and the gas-wash bottle contained the same amount of CO_2 (≈250ppm), a similar percentage of behaviours was recorded with around 40% of flies attracted.

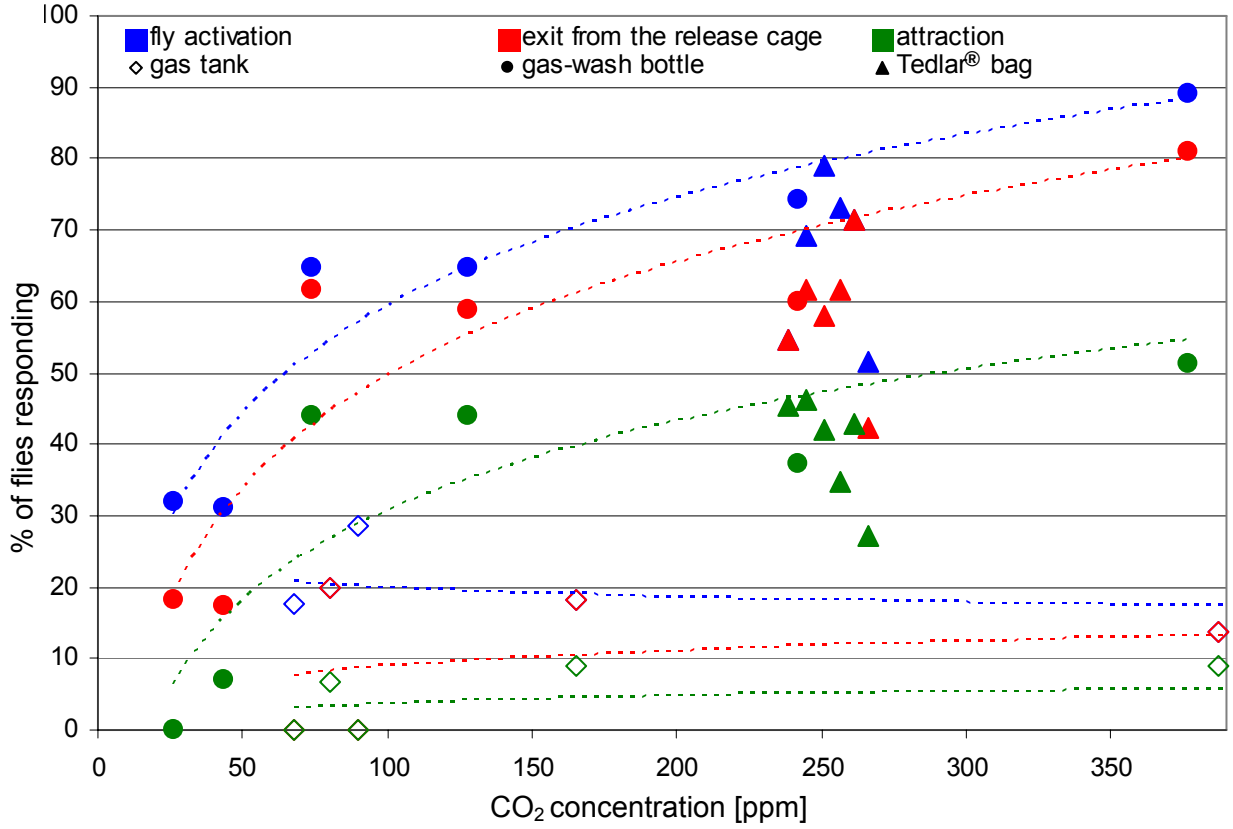

Figure 3. Behavioural responses of *G. brevipalpis* to stimulation with comparable CO_2 concentration in breath as delivered from a 1L gas-wash bottle and a Tedlar® bag, and from a gas tank.

The behavioural criteria recorded were: *activation* (blue figures), the fly moved in the cage, *exit* (red figures), the fly flew from the release cage; *attraction* (green figures), the fly made a directed flight in the plume of breath to within 10cm of the source.
Each point represents the result of one sample with empty diamonds for gas tank, filled square for gas-wash bottle and filled triangle for Tedlar® bag.
For more details see legend of *Figure 1*, *Table 1* and *Material & Methods*.

As it is shown in the table 1, many other compounds are present in breath and we hypothesised that some of them can be perceived by tsetse flies.

Aldehydes provide a class of products regularly identified in breath. C6 to C11 aliphatic aldehydes evoke electroantennogram (EAG) response from *G. pallidipes* antennal receptor cells when injected at 100ng onto a gas chromatographic column (Fig. 4). The highest antennal response was evoked by decanal, followed by undecanal and nonanal, with hexanal, heptanal and octanal eliciting only lower EAG responses.

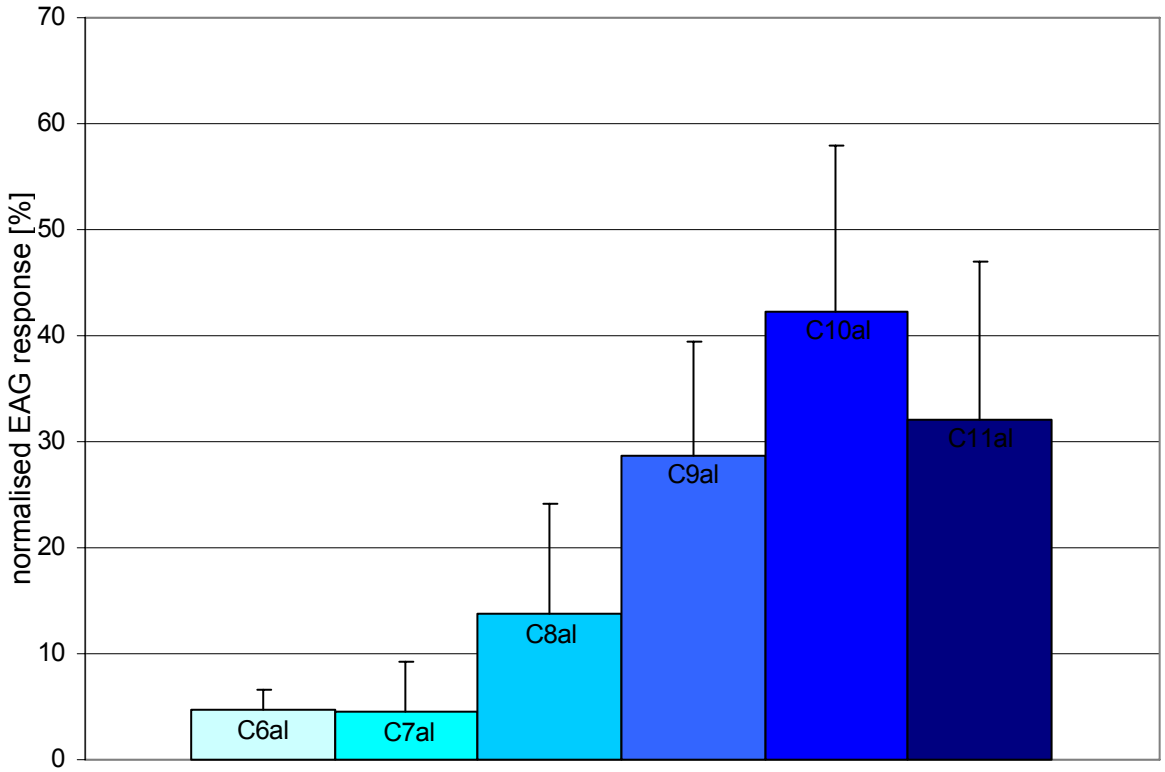

Figure 4. Electroantennogramme responses of *G. pallidipes* to 100ng doses of synthetic aldehydes injected onto a gas chromatographic column.

The antenna responses were recorded during elution from the gas chromatographic column of the aldehydes mixture composed of hexanal (*C6al*, n=3 antennae), heptanal (*C7al*, n=2), octanal (*C8al*, n=5), nonanal (*C9al*, n=5), decanal (*C10al*, n=5) & undecanal (*C11al*, n=5).
The antennal responses were normalised using an odour puff over 1µg 1-octen-3-ol in a stimulus syringe as reference at the start and end of each chromatographic analysis.

However, gas chromatography linked antennogram recordings with lower doses of these aldehydes showed no difference of threshold between the heaviest aldehydes tested (nonanal, decanal and undecanal) on *G. pallidipes* antenna (Fig. 5). Octanal and heptanal, even though less extensively tested, showed much lower antennal responses.

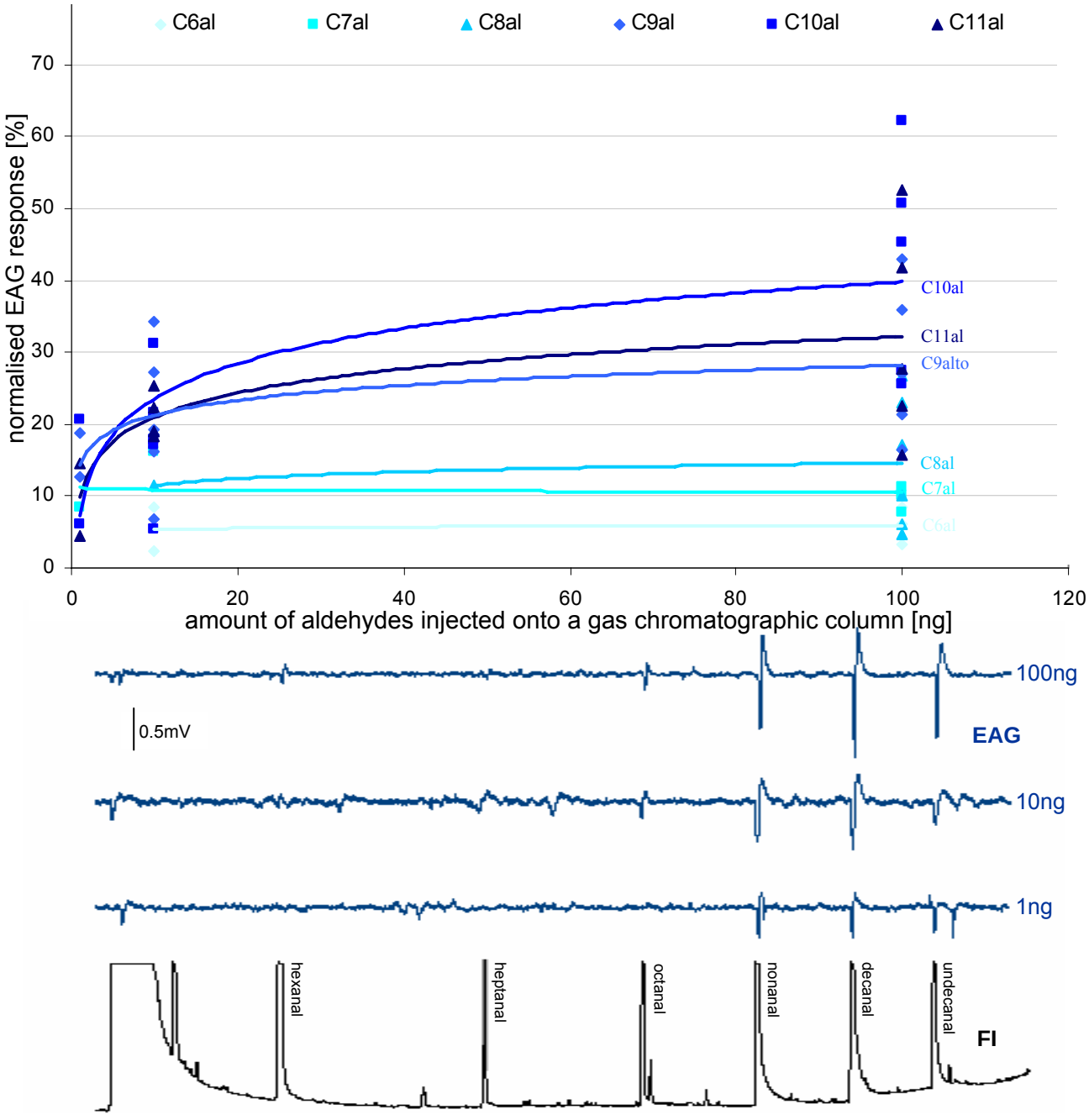

Figure 5. Electroantennogramme responses of *G. pallidipes* to different amounts of synthetic aldehydes presented to the antenna by gas chromatography coupled EAG recording.

The antenna responses were recorded during elution from the gas chromatographic column of different doses of an aliphatic aldehyde mixture containing: hexanal (C6al, n=5 antennae), heptanal (C7al, n=5), octanal (C8al, n=7), nonanal (C9al, n=13), decanal (C10al, n=12) & undecanal (C11al, n=11).
The antennal responses were normalised using a puff of 1µg of 1-octen-3-ol in a stimulus syringe as reference at the start and end of each analysis. Each colored point on the graph represents one response of an antennae to an amount of compound.

Compounds from plants such as terpenes passing untransformed through the digestive system and ketones resulting from the transformation of plant tissues or terpenoids, can be found in breath depending on the food source of the animal. A mixture of terpenes comprising iso-caryophyllene, β-farnesene and germacrene D elicited EAG responses (Fig. 6).

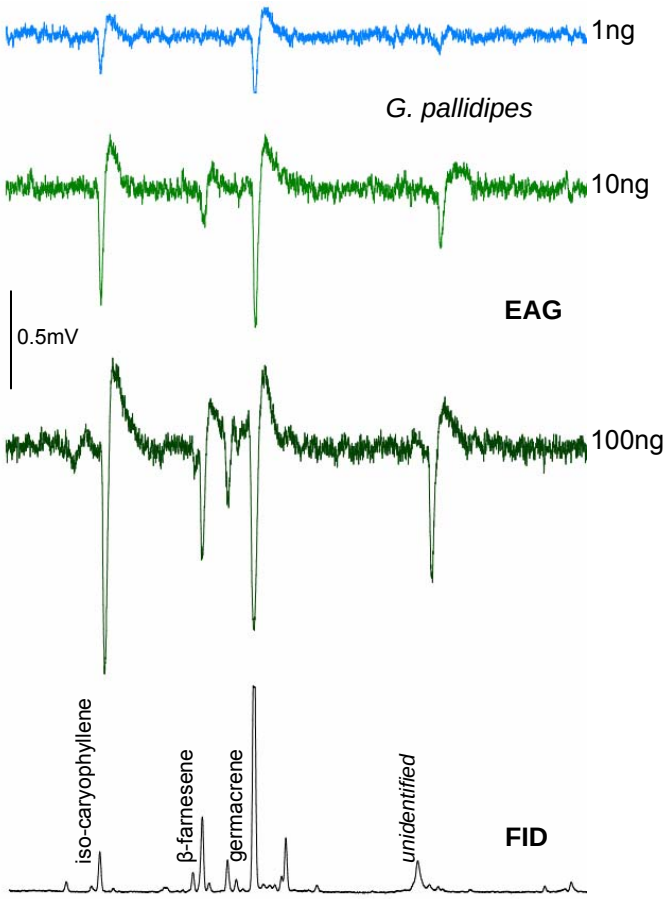

Figure 6. EAG of *G. pallidipes* to a mixture of terpenes presented at different amounts to the antenna by gas chromatography coupled EAG recording.

The lower trace is the flame ionisation detector (FID) response of the chromatograph and the upper traces are the EAG responses (EAD) of the antenna olfactory cells generated during elution of the test mixture from the gas chromatographic column.
Scale bar in mV provides a measure of the depolarisations recorded.

Plotting the amplitude of the EAG responses generated against the quantity of compound eluting over the antenna, a measure of the relative sensitivity of the *G. pallidipes* olfactory system to terpenes and ketones can be obtained (Fig. 7). Even though there were large variations in the antennal responses between recordings *G. pallidipes* showed the highest sensitivity to β-caryophyllene and iso-caryophyllene, lesser to 3-octen-2-one and germacrene D, and lowest responses to β-farnesene among the compounds tested.

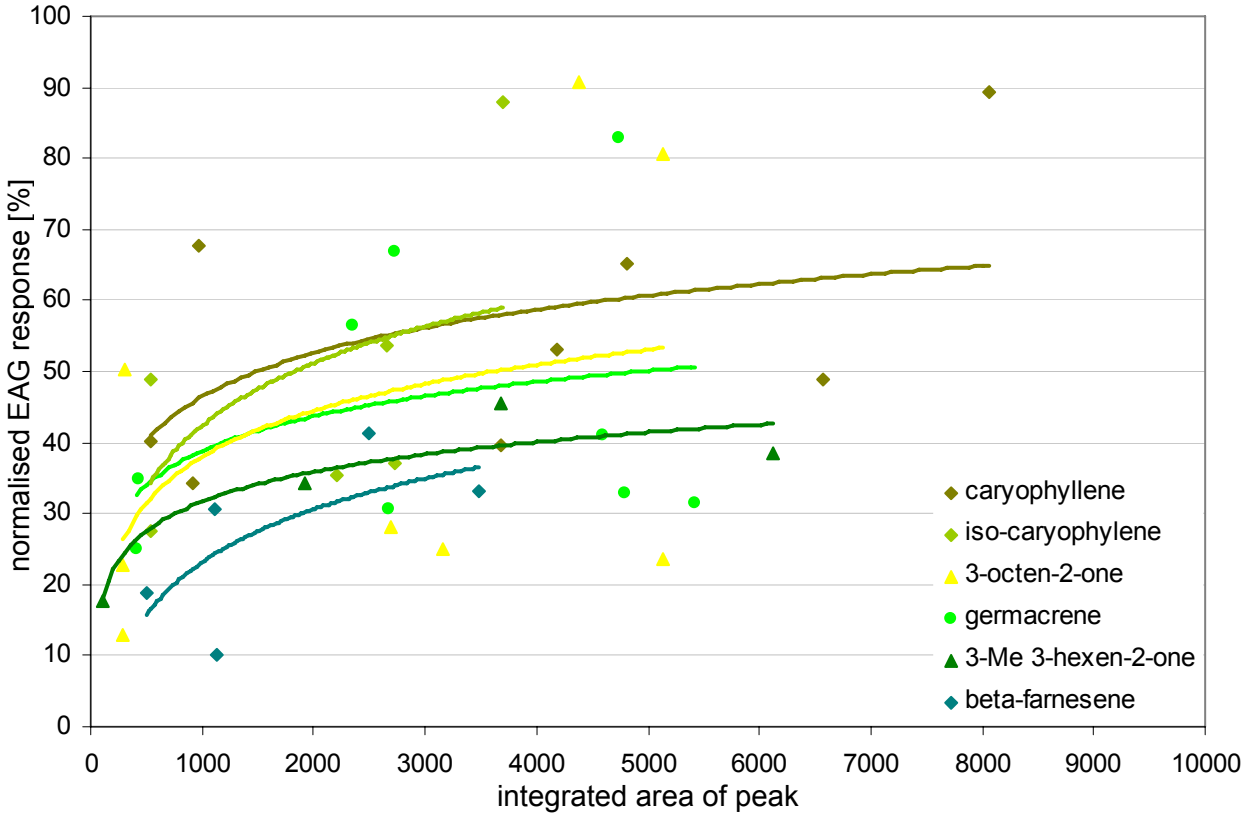

Figure 7. Electroantennogramme responses of *G. pallidipes* to different amounts of synthetic terpenes and a ketone presented to the antenna by gas chromatography coupled EAG recordings.

The EAG responses were recorded during elution from the gas chromatographic column of the different mixtures of products: caryophyllene (n=11), iso-caryophyllene (n=6), 3-octen-2-one (n=8), germacrene (n=9) & β-farnesene (n=6). Each point on the graph represents one response of an antennae to an amount of compound.
The antennal responses were normalised using a puff of 1µg of 1-octen-3-ol in a stimulus syringe as reference at the start and end of each analysis.

Other ketones and terpenes were tested by the EAG on antennae of *G. brevipalpis*, *G. fuscipes* and *G. pallidipes*. Most of the EAG responses were the same for the 4 compounds tested in the 3 tsetse species (Fig. 8). The EAG increased as a function of dose with the strongest responses for β-cyclocitral and 3-octen-2-one in all three species, even if not very different from the EAG responses to 2-undecanone and 6-methyl-5-hepten-2-one. *G. brevipalpis* showed a very high sensitivity to 3-octen-2-one in that the EAG response started to increase when 100ng of compounds were put on the paper. The thresholds recorded for EAG responses by *G. pallidipes* were higher requiring 10µg of β-cyclocitral and 3-octen-2-one in the stimulus cartridge to obtain EAG responses different from the solvent control.

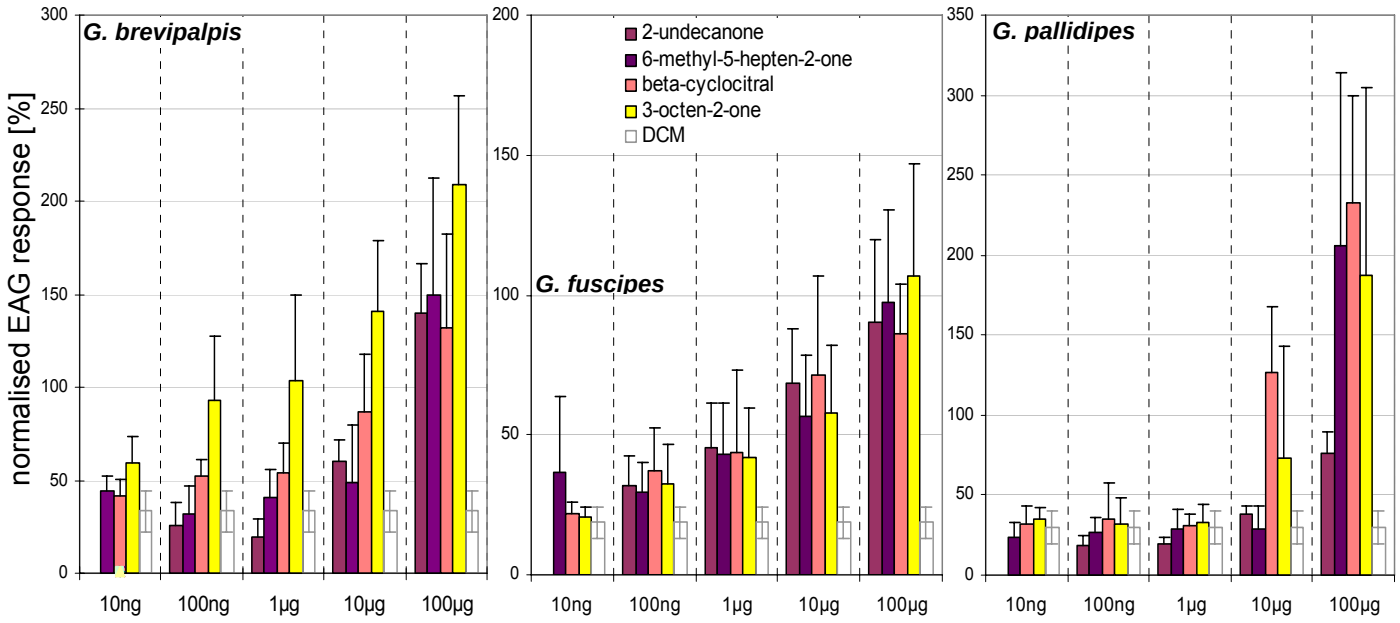

Figure 8. EAG responses of *G. brevipalpis*, *G. fuscipes* & *G. pallidipes*, to synthetic ketones and a terpene presented at different concentrations in air passing over the antenna.

The EAG responses of *Glossina brevipalpis* (n≥2 & ≤10), *G. fuscipes* (n≥4 & ≤15) & *G. pallidipes* (n≥2 & ≤15) were recorded to an air puff containing the odour 3-octen-2-one, β-cyclocitral, 6-methyl-5-hepten-2-one & 2-undecanone diluted in dichloromethane (DCM) at 5 different doses ranging from 10ng to 100µg in the stimulus syringe. The EAG responses were normalised using an odour puff from 100ng 1-octen-3-ol in the stimulus syringe as reference at the start and end of each recording period. The response to pure DCM (n=16, 30, 39 for each spp. respectively) provides an estimation of the response threshold for each compound.

Other noteworthy compounds which can be found in breath are sulfide products. EAG responses of *G. fuscipes* and *G. pallidipes* were recorded to dimethyl disulfide and dimethyl trisulfide detected in the rumen and which are exhale by ruminants during eructation. The 2 tsetse fly species show EAG responses to dimethyl trisulfide in a dose dependent manner (Fig. 9 & 10). The antennal receptor cell responses are specific to this substance as no response was recorded for dimethyl disulfide at any of the 3 different amounts tested. Furthermore, neither dimethyl sulfide nor dipropyl sulfide elicited EAG responses from *G. pallidipes* (electroantennograms not shown on *G. pallidipes*), the latter sulphides were not tested with *G. brevipalpis*.

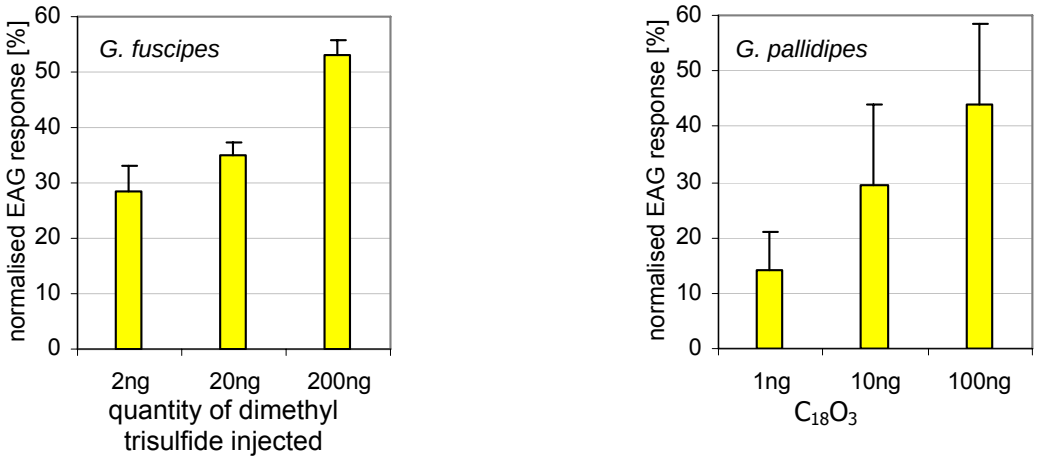

Figure 9. EAG responses of *G. pallidipes* and *G. fuscipes* to different amounts of dimethyl trisulfide eluting from a gas chromatographic column.

The compound was tested at 3 different amounts in the 1µL of solution injected: at 1ng, 10ng & 100ng for *G. pallidipes* (with n=4, 6 and 9 antennae respectively) and at 2ng, 20ng & 200ng for *G. fuscipes* (with n=2, 3 and 2 respectively). The antennal responses were normalised using an odour puff from 1µg of 1-octen-3-ol in a stimulus syringe as reference at the start and end of each analysis.

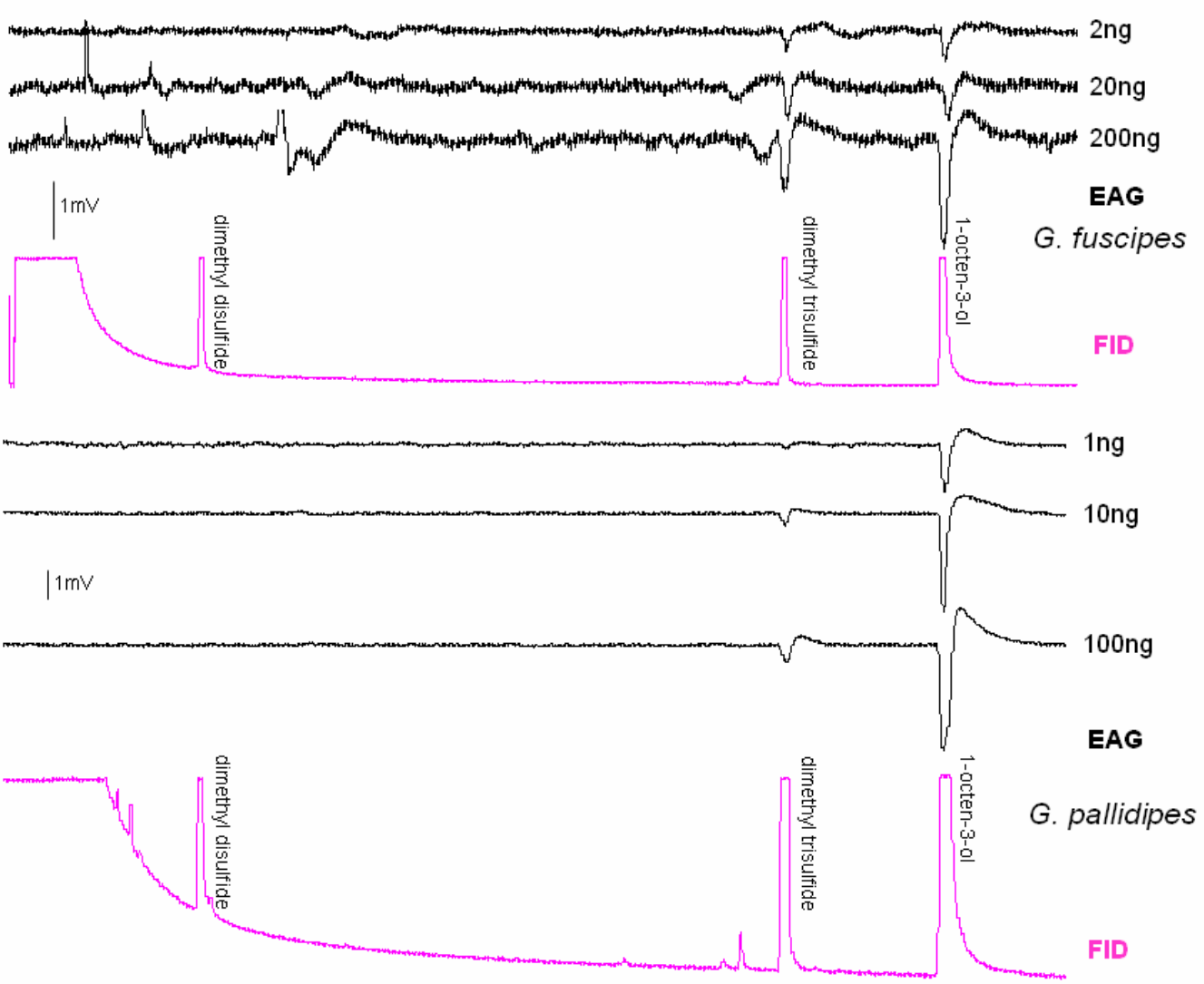

Figure 10. EAG responses of *G. pallidipes* and *G. fuscipes* to different amounts of dimethyl disulfide, dimethyl trisulfide and 1-octen-3-ol eluting from a gas chromatographic column.

The pink traces are the flame ionisation detector (FID) responses of the chromatograph and the black traces are the EAG responses generated during elution of the test products from the gas chromatographic column. The compounds were tested at 3 different amounts in the 1µL of solution injected: at 1ng, 10ng & 100ng for *G. pallidipes* and at 2ng, 20ng & 200ng for *G. fuscipes*. The scale bars in mV provide a measure of the depolarisations recorded.

Another molecule, benzothiazole, which contains a benzene ring fused with a thiazole ring, was tested by EAG using antennae of *G. pallidipes* and *G. brevipalpis* (Fig. 11). The amount of benzothiazole injected in the GC linked EAG recordings was different for *G. pallidipes* and *G. brevipalpis* so it was not possible to compare the dose responses of the two species. However, at 100ng, the EAG responses of the two species were the same, with a slightly higher response for benzothiazole than for dimethyl trisulfide.

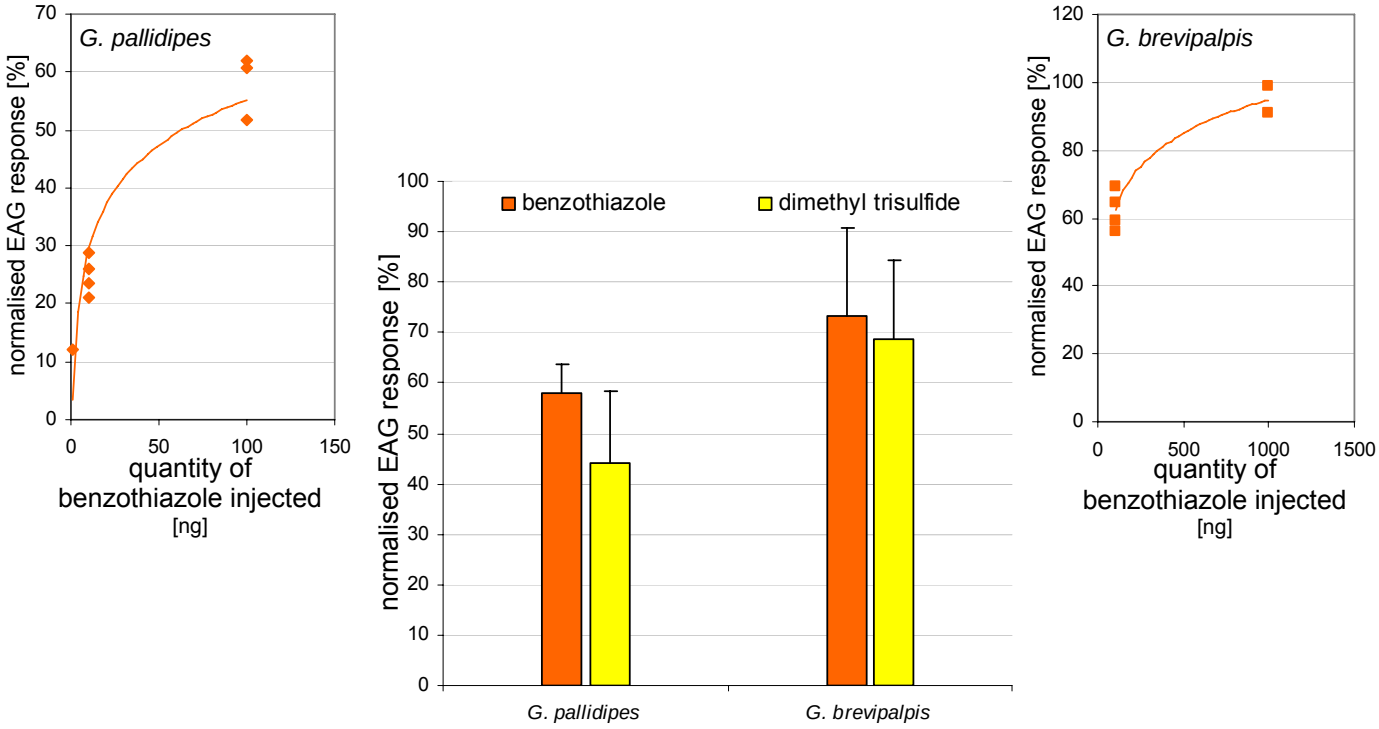

Figure 11. EAG responses of *G. pallidipes* and *G. brevipalpis* to benzothiazole and dimethyl trisulfide presented at different amounts to the antennae by gas chromatography coupled EAG recording.

The antenna responses were recorded during elution from the gas chromatographic column benzothiazole and dimethyl trisulfide. The antennal responses were normalised using an odour puff from 1µg of 1-octen-3-ol in a stimulus syringe as reference at the start and end of each analysis.
The upper graphs on left and right depict the EAG responses of *G. pallidipes* and *G. brevipalpis* to different amount of benzothiazole.
The central histograms show the EAG response of *G. pallidipes* and *G. brevipalpis* to 100ng benzothiazole and dimethyl trisulfide eluting from the chromatographic column.

Discussion

G. brevipalpis was attracted by human breath in our wind tunnel. Moreover, the behavioural results of stimulation with breath dispensed from a gas-wash bottle showed that the flies were affected by breath dilution, it would appear linked to the decrease in CO_2 released. Tsetse flies are known to possess CO_2-sensitive receptor cells with a threshold around 0.03% (Bogner 1992), and Evans & Gooding (2002) obtained significant activation with a CO2 levels fluctuating 0.0051% above background. Even if CO_2 alone permits to increase trap captures of morsitans flies (Rennison and Robertson 1960; Vale and Hall 1985) and palpalis flies (Frezil & Carnevale, 1976; Galey et al., 1986), *G. brevipalpis* did not respond well to stimulation with CO_2 alone in our apparatus. But as already reported for ticks (McMahon and Guerin 2002), mosquitoes (Healy and Copland 1995), biting-fly (Warnes and Finlayson 1985) and tsetse flies (Warnes 1990), human breath acts as a much stronger attractant than CO_2 alone. On the other hand, as shown by Warnes & Finlayson (1985) and Green (1993), breath without CO_2 generates low activation rate. Torr (1990) and Zollner *et al.* (2004) believed that because CO_2 falls rapidly to background levels after dispersing from a potential host, it is likely to be only a close attractant. Evans & Gooding (2002) found that adding humidity to a turbulent plume of heated air and CO_2 significantly increased the behaviour of *G. m. morsitans*. In fact, CO_2 spontaneously interconverts between CO_2 and H_2CO_3 in water, and so we can hypothesise that breath which contains 6% of water vapour can improve the CO_2 travel by forming a canal which reduce the dispersion of CO_2 and other polar compounds. Elevated CO_2 would especially indicate that a potential host is nearby, and other less diffusive compounds would be involved in the attractiveness at longer distances.

The other components of breath can be divided in two groups. There is first of all a “small core” of breath volatile organic compounds which are probably produced by metabolic processes common to most vertebrate hosts. The second set of volatiles products in breath will depend on the animal and its food. For example, ruminant breath contains anaerobic products formed in the rumen by hydrolysis and fermentation of plant polymers by micro-organism (Elliott-Martin et al., 1997; Spinhirne et al., 2004). An overview of the most recurrent and interesting molecules cited in the literature is presented in table 1. Most of them have never been studied extensively in the context of host location by tsetse flies, and for this reason EAG recordings were made from tsetse flies of different subgenera to some of these products.

Aldehydes are mainly formed after oxydation of a primary alcohol, and can be converted to a carboxylic acid or an alcohol. They constitute a family of products of cattle already tested during a field study of Vale (1980) with various other chemicals to attract tsetse flies. He tested the

effect on trap attraction of C_1 to C_7 aldehydes and their branched isomers (2-methyl C_4 and 3-methyl C_5) added to CO_2. The doses applied were different between aldehydes (0.03 to 6g/h) and high (20 to 40'000 times more than attractants such *p*-cresol used in the field nowadays), but this author obtained significant catch increases with formaldehyde, propionaldehyde and butyraldehyde. However these three low molecular weight molecules are not the most predominant aldehydes detected in breath. It is the C_6 to C_{12} aliphatic aldehydes that predominate in skin emanation of humans (Bernier et al., 2000; Curran et al., 2005) and body odour of buffalo, ox, waterbuck (Gikonyo et al., 2002), giraffe (Wood & Weldon, 2002), bontebok and blesbok (Burger *et al.*, 1999b). Steullet & Guerin (1994) detected two receptor cells sensitive to hexanal, heptanal and nonanal within the Haller's organ capsule of the tarsus of *Amblyomma variegatum*. From the C_7 to C_{12} aldehydes detected in cow rumen fluid by Jeanbourquin (2005), octanal, nonanal and decanal evoked EAG responses from *Stomoxys calcitrans* antennal receptor cells, with a higher response to decanal than to other products tested. Du & Millar (1999) obtained EAG responses linked to the dose of nonanal in *Culex quinquefasciatus*. In our study, C_9 to C_{11} aldehydes evoked the best EAG responses in *G. pallidipes*. The EAG responses is quite similar between nonanal, decanal and undecanal, a finding that matches well with single sensillium recordings made by Guerenstein & Guerin (2001) on *Triatoma infestans* where C_7 to C_9 aldehydes evoked similar responses from receptor cells in basiconic sensilla. However, among these three aldehydes, only nonanal was able to induce an increase in walking speed of the triatomine on a servosphere. With the same methods used for studying *T. infestans*, McMahon & Guerin (2002) found that the tropical bont tick *A. variegatum* was attracted to small doses (10ng on filter paper) of hexanal and nonanal. Similar result was obtained by Du & Millar (1999) who documented that nonanal was attractive to *Cx. tarsalis* and *Cx. quinquefasciatus*, and served as an oviposition stimulant for *Cx. tarsalis*. Field tests on tsetse flies showed that decanal alone improved capture of *G. brevipalpis* in modified leg panel traps, and when added to traps baited with the POCA mixture (3n-propyl phenol + 1-octen-3-ol + p-cresol + acetone), it permitted to significantly increase the captures of *G. pallidipes* and *G. brevipalpis* (IAEA-TECDOC 2003). Clearly the effects of aldehydes such as nonanal and decanal affecting the behaviour of different haematophagous arthropods need to be further investigated on tsetse fly behaviour.

Ketones are widespread in vertebrate odour and arise from different biosynthetic pathways. An example is 3-octen-2-one which is an intermediate in the synthesis of 2,3-octandione, highly characteristic of a pasture diet in cattle and formed during the mastication of grass from linoleic and linolenic acid (Young, Berdague et al. 1997). Similar pathways of fatty acid degradation give rise to acetone from decarboxylation of ketones body. On the other hand,

6-methyl-5-hepten-2-one is an isoprenoid derivate and can be produced during oxidative cleavage of terpenes such as the lycopene formed by 8 isoprene units (Goff and Klee 2006). If ketone levels in host is linked to starvation or possibly trypanosomiasis infection as proposed by Torr *et al.* (1995), they may provide an interesting cue for increasing the detection and attraction of tsetse flies to a weak host less able to defend itself. Among this family of compounds, acetone is the most often encountered in host animals for tsetse flies. This molecule is efficient and widely used to increase traps attraction, it causes problems of maintenance due to its high evaporation rate. Moreover Den Otter et al. (1988) found that it showed the highest EAG threshold in five tsetse fly species compared to the heavier 4-heptanone and 3-nonanone. These authors calculated that 10^3 to 10^4 times greater amounts of acetone are needed to obtain EAG responses similar to the ones elicited by 1-octen-3-ol. Willemse (1991) replaced acetone by butanone in field experiments and reported that the attraction of *G. m. centralis* was the same whereas the release rate of butanone was lower. As butanone is found in cow breath (Tab 1), we may postulate that heavier ketones of animal or plant origin should permit to obtain similar effects. 6-methyl-5-hepten-2-one was identified in body odours of cattle and waterbuck, and was moreover detected in cow rumen fluid (Gikonyo et al., 2002; Jeanbourquin, 2005). Because ruminants regularly void rumen metabolites through eructation, we can suggest that this molecule is found in trace amount in breath. This product has been identified as a chemostimulant for the other major cattle fly pests *S. calcitrans*, *Haematobia irritans*, *Musca autumnalis*, *Hydrotaea irritans* and *Wohlfahrtia magnifica* (Birkett et al., 2004; Jeanbourquin, 2005). Our EAG study shows that tsetse fly species representing the 3 subgenera are able to perceive this molecule but at a relatively high threshold. Moreover, this product was tested in the field by Vale (1980): when 6-methyl-5-hepten-2-one was added to CO_2, it causes a slight increase in trapping of Glossina spp. of the *morsitans* group. Like 6-methyl-5-hepten-2-one, 2-undecanone is found on the scent of various animals such as cattle, waterbuck, bontebok and blesbok (Burger *et al.*, 1999a; Gikonyo *et al.*, 2002; Jeanbourquin, 2005), and has been described as a chemostimulant for haematophagous arthropods such as *Cx. quinquefasciatus* and *S. calcitrans* (Du & Millar, 1999; Jeanbourquin, 2005). 2-undecanone elicited EAG responses from the 3 tsetse fly species examined in this study, with particularly good responses from *G. fuscipes*. The third ketone tested, 3-octen-2-one, evoked strong EAG responses from *G. brevipalpis*, equivalent to the response evoked by 1-octen-3-ol at 100ng. Linoleic and linolenic acids are common precursors for 3-octen-2-one and Saini *et al.* (1989) drew the conclusion that good 1-octen-3-ol analogues should posses a non rigid functional end and an odd carbon number alkyl chain. As 3-octen-2-one molecule corresponds to this, it may explain the strong EAG responses recorded to this compound from tsetse fly antennae of

the 3 species in this study. 3-octen-2-one may bind to the same receptor site as 1-octen-3-ol on the antennal receptor cells such that 3-octen-2-one would not improve responses to traps already baited with 1-octen-3-ol. Even though tsetse flies of the three subgenera are able to detect 6-methyl-5-hepten-2-one, 2-undecanone and 3-octen-2-one no behavioural tests has ever been performed on tsetse flies with 3-octen-2-one and 2-undecanone.

Terpenes are a large and varied class of molecules, primarily synthesised from units of isoprene by a wide variety of plants. Most of them are inducible volatile organic compounds produced by plants after foliage damage, as happens during plant feeding and digestion by vertebrate hosts. Because most of these compounds pass untransformed through the digestive system, they were found in bovid rumen fluid and breath (Syed, 2002; Jeanbourquin, 2005), but their presence in vertebrate breath largely depends on the plants the animal had fed on. Mosquitoes must regurlarly seek plants as they depend on nectar as a source of energy for flight. Bowen (1992) found that *Cx. pipiens* females can detect terpenes using their antennal receptor cells, and behavioural assays suggested a possible role for these compounds in nectar feeding by mosquitoes (Healy and Jepson 1988). Even if tsetse flies are strictly haematophagous, plants provide shelter in all biotopes they occupy, permitting flies to mature, digest and gestate under equitable conditions, and also provide females with places protected from direct sunlight for larval deposition. Our EAG recordings from the antennae of 3 tsetse species from different African habitats show that they are able to perceive β-caryophyllene, iso-caryophyllene, germacrene D, β-farnesene and β-cyclocitral. The responses were linked to dose with the highest sensitivity being shown to 2 stereoisomer of caryophyllene (beta- and iso-), and a good sensitivity for germacrene D. These results correspond well with previous EAG recordings from tsetse flies made with different essential oils from plants (Mohottalage, 2002; Syed, 2002). Moreover, Syed & Guerin (2004) documented that the invasive plant *Lantana camara*, and in particular a mixture of 7 compounds including 4 terpens extracted from it, attract 3 different tsetse fly species. As compounds from plants used in seeking a refugium by tsetse flies can also occur in hosts, these insects make parsimonious use of chemostimuli.

Because of their uncommon sulfur atom, sulfides are an interesting group of products to treat in the search for biologically active compounds for tsetse flies. They mainly come from decomposition of sulfur-containing material by bacterial action. For example, *Enterobacter cloacae* is a commensal microbe found in animal and human intestines as well as in milk or faeces and this bacterium can produce dimethyl disulphide or dimethyl trisulfide (Schöller, Molin et al. 1997). Thereby, sulfur compounds occur in the rumen (Jeanbourquin and Guerin 2007), livestock wastes (Mackie, Stroot et al. 1998), human skin (Bernier, Kline et al.

2000) or interdigital glands of reindeer (Nilssen, Age Tommeras et al. 1996). As most of the sulfur molecules have the amino acids cystein or methionine as precursors, they are always found at low amounts. Methionine is an essential amino-acid, and compounds arising from its transformation such as methanethiol which is oxidized into dimethyl disulfide and dimethyl trisulfide (Weimer, Seefeldt et al. 1999), may provide cues of a host for heamatophagous arthropods. Sulphide-receptors have already been identified in various arthropods such as *A. variegatum* (Steullet and Guerin 1992), *M. domestica* (Cossé and Baker 1996), *S. calcitrans* (Jeanbourquin and Guerin 2007), *Hypoderma tarandi* and *Cephenemyia trompe* (Nilssen, Age Tommeras et al. 1996). In this study we have found that different species of tsetse flies are also able to perceive this family of substances. However, the detection threshold for dimethyl trisulfide in *G. pallidipes* and *G. fuscipes* is higher than that of other flies such as *S. calcitrans* and *M. domestica* (Cossé & Baker, 1996; Jeanbourquin & Guerin, 2007), and, unlike *M. domestica*, tsetse flies are not able to perceive dimethyl disulfide as reported here. Moreover, different studies have shown that sulphides present in breath (Tab 1) and human skin emanations (Bernier, Kline et al. 2000) can modify the behaviour of haematophagous arthropods. For example, *Aedes aegypti* and *Cx. nigripalpus* were attracted by dimethyl disulfide presented on its own in an olfactometer, and whereas dimethyl trisulfide did not attract these species, it increased the number of landings by *Cx. quinquefasciatus* and *Cx. nigripalpus*, whereas dimethyl disulfide did not (Allan, Bernier et al. 2006). Dimethyl trisulfide used on its own attracted *S. calcitrans* in a wind tunnel (Jeanbourquin and Guerin 2007), *Cx. tarsalis* to sticky traps (Du and Millar 1999) and 7 species of *Calliphoridae* to baited traps (Nilssen, Age Tommeras et al. 1996). To our knowledge, effects of sulphide compounds on tsetse flies have never been tested in the field.

CO_2 is an attractant for haematophagous arthropods such as tsetse flies, but its action may occur only at a relatively short range. We conclude that in our wind-tunnel, *G. brevipalpis* used other compounds present in breath to reach the source. Among these products, potential products belong to different chemical families which have not been extensively tested in the field. Substances such as decanal, caryophyllene or benzothiazole that elicite EAG responses from different species of tsetse flies, merit attention.

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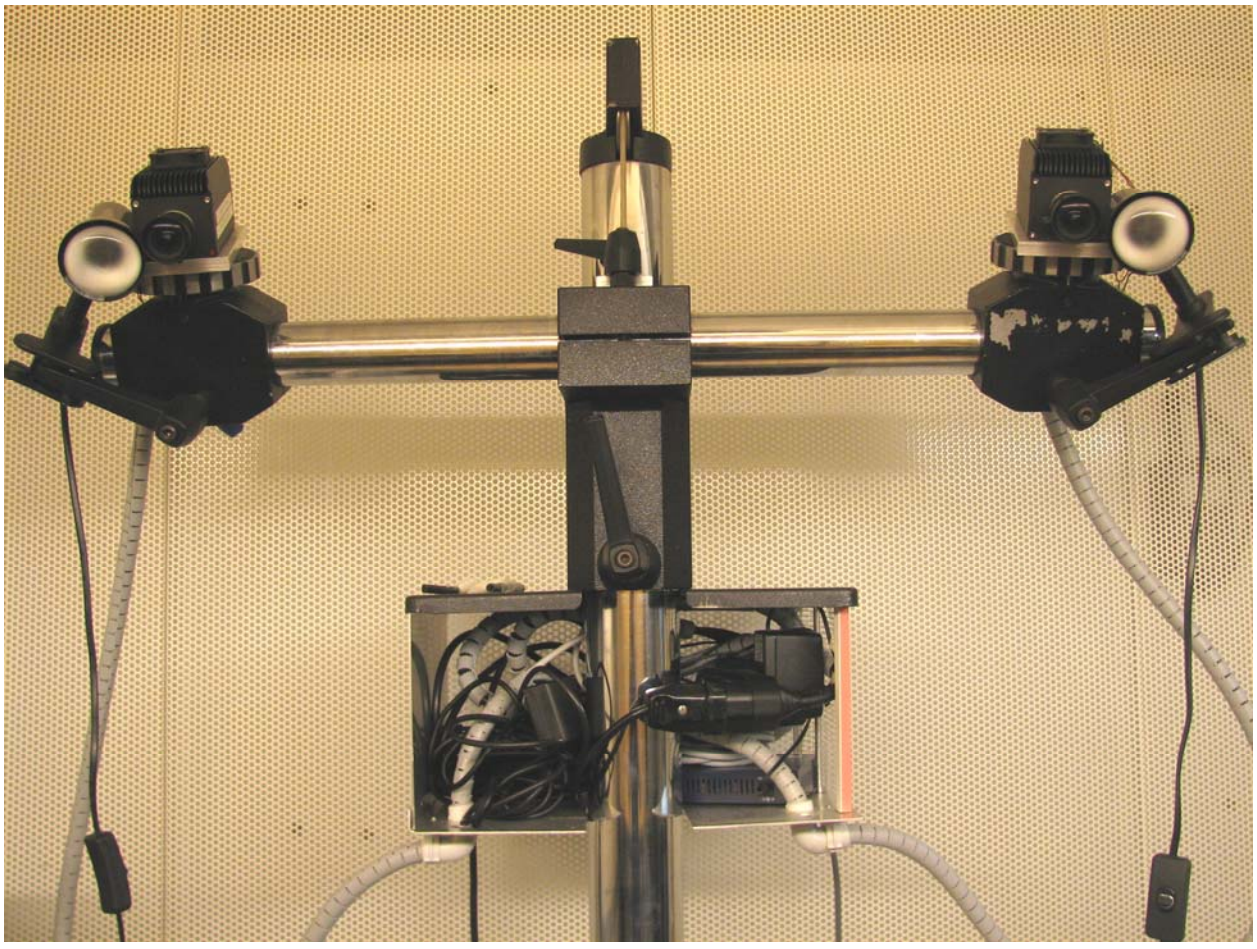
Chapter 4



Sensory responses and attraction of tsetse flies *Glossina* spp. to alkanes

Abstract

Although alkanes are well-known volatile metabolites present in vertebrate breath, little attention has been paid on the influence of this family of compounds on insect behaviour. Using electroantennogram recordings from *G. pallidipes* and *G. brevipalpis* antennae, we demonstrated that tsetse flies are able to perceive C_5 to C_{11} alkanes with a sensibility slightly higher than for acetone, a known tsetse fly chemostimulant. Moreover, flight tests in a wind tunnel showed that hexane or heptane dispensed has a synergistic effect on *G. brevipalpis* attraction when mixed with the attractant mixture used in the field made up of 3*n*-propyl phenol, 1-octen-3-ol and *p*-cresol. As acetone is an efficient component of tsetse fly field baits but problematic to maintain due to its high volatility, we discuss the possibility of substituting it by heptane which induced similar behavioural effects on *G. brevipalpis* in our wind tunnel trials but at a lower release rate.

Key words : tsetse flies, olfaction, , electroantennogram recordings, wind-tunnel, alkanes, breath.

Introduction

Tsetse flies (Diptera, Glossinidae) are present in different ecosystems in sub-Saharan Africa: open savannah woodlands (*morsitans* group with 5 spp.), forests (*fusca* group with 11spp.) and riverine and lakeshore habitats (*palpalis* group with 5 spp.) (Jordan 1986). Both sexes feed on blood during which they can transmit trypanosomiasis to the host. A number of studies mostly concentrated on the *morsitans* group have been conducted with the aim to reduce insect populations using visual traps and targets. This gave rise to the development of attractive baits used with these traps incorporating 4 volatiles isolated from host odour: 3*n*-propylphenol, 1-octen-3-ol and *p*-cresol at a ratio 1:4:8 dispensed from a polyethylene sachet plus acetone evaporating from glass bottle (Vale, Hall et al. 1988). Even though flies are attracted to a distance of 60-120m downwind to such devices they require regular maintenance because of the high release rate of acetone.

Acetone is, like CO_2, a small molecule detected from breath that attracts tsetse flies (Vale 1980). Little attention has been paid to the influence of other commonly occurring small molecules in breath such as alkanes (Phillips *et al.*, 1999; Spinhirne *et al.*, 2004). However, there is ample evidence in the literature that small polar and apolar molecules can influence insect behaviour. Most of the time, the effect of these molecules is seen from a negative point of view as they are frequently used as solvents, thus providing a source of potential bias in studies. For example, Healy & Jepson (1988) recorded landing response by *Anopheles arabiensis* to their negative pentane control, but they did not draw any conclusion from this. On the contrary, Bernier *et al*. (2003) cited dichloromethane as one of the compounds synergising lactic acid in attracting *Aedes aegypti*. Jewett & Bjostad (1996) who observed the same attractive effect of dichloromethane on diabroticite larvae have shown how this solvent could mimic CO_2 based on structural similarity. This conclusion supports the results of Bernier *et al.* (2003) with *Ae. aegypti* known to be attracted by CO_2. Otherwise little is still known about the influence of these small molecules on insect behaviour. Alkanes in breath mostly result from lipid peroxydation and so can be synthesised naturally in complex biological systems (Riely, Cohen et al. 1974) via the oxidative degradation of lipids that gain an –OOH radical forming a lipid hydroperoxide. The most probable reaction is then cleavage between the 2 oxygens of the R-OOH group, followed by

cleavage of a C-C bond on either side of the carbon carrying the oxygen atom (Fig. 1). In the example depicted, linoleic acid molecule liberates pentane. Via another pathway, linoleic acid can give rise to 1-octen-3-ol, which elicits a powerful response from antennal receptor cells of tsetse flies.

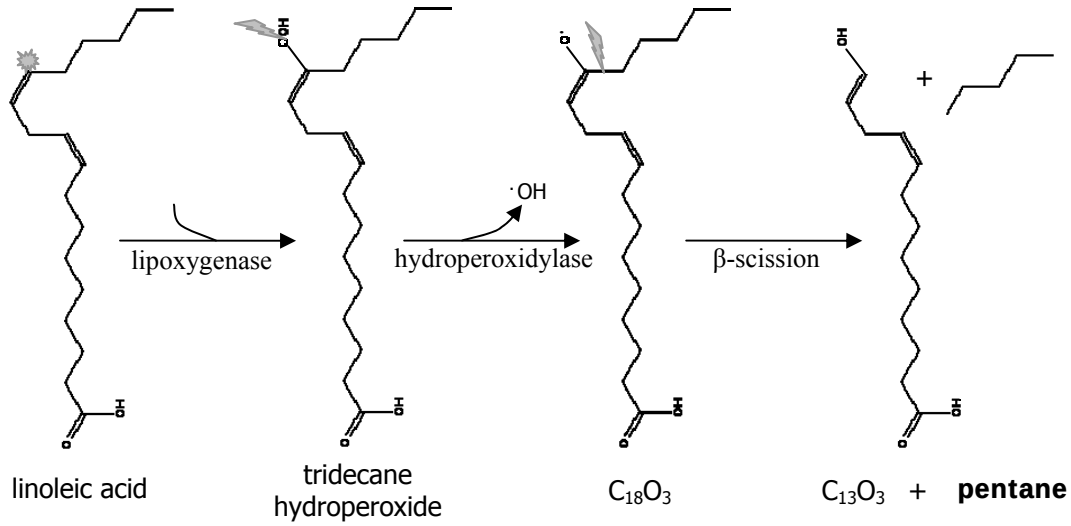


Figure 1. Pathway of pentane formation from linoleic acid hydroperoxyde decomposition.

Lipid peroxydation occurs when a cell membrane is damaged, such as during the mastication of plants by the herbivore hosts of tsetse flies. Moreover, carboxylic acids, the preponderant end products of the rumen are continually evacuated with fermentation gases, usually after travelling into the lungs. Through residence in the aerobic environment of the lungs, carboxylic acids are eventually transformed. In this way, decanoic acid and undecanoic acid can contribute via lipid peroxydation to respectively octane and heptane in breath. Alkanes ranging from C_5 to C_{10} are well known constituents of breath (Phillips *et al.*, 1999; Spinhirne *et al.*, 2004), and therefore merit testing as products that may modify tsetse fly behaviour.

The response of olfactory receptor cells of two tsetse fly species *G. pallidipes* and *G. brevipalpis*, belonging to two different subgenera were quantified to estimate the sensitivity of their olfactory systems to different alkanes and to compare this with acetone. Then, behavioural tests on free flying *G. brevipalpis* were made in the wind tunnel to quantify any behavioural responses to hexane, heptane and octane compared to acetone. Inclusion of compounds used to attract tsetse flies in the field (3*n*-propylphenol, 1-octen-3-ol and *p*-cresol) in these tests permitted to consider the substitution of acetone in traps to capture tsetse flies of the *fusca* group.

Materiel & Methods

Insects

Tsetse flies were held in environmental conditions similar to the those described in the chapter 2.

Behavioural experiments in the wind tunnel

The responses of 20 female and 20 male *G. brevipalpis* to alkanes and acetone were recorded in the wind tunnel. Charcoal-filtered air at 5mL.min^{-1} passed through a 1L gas-wash bottle containing nothing or a polyethylene sachet (4×4cm, 150µm film thickness) loaded with 1mL of pure 1-octen-3-ol or 1mL of a mixture of 3*n*-propylphenol, 1-octen-3-ol and *p*-cresol at a ratio 1:4:8 respectively, and 500mL.min^{-1} passed through a 2nd gas-wash bottle filled with 5mL of pure acetone, hexane, heptane or octane. The two streams were mixed and introduced via a 2mm i.d. teflon tube linked to the upright aluminium tube at the upwind end of the wind tunnel and plume generator (see chapter 2 for details). The negative control consisted of charcoal-filtered air dispensed at 0.5L/min directly from the stimulus controller (see *chapter 2*) to the plume generator. The compounds used for these experiments are listed in table 1.

The wind tunnel and the behavioural recording methods used for these experiments were similar to those described in the chapter 2.

Electroantennogram recordings

The methods used to record electroantennogram (EAG) responses to test stimuli were as described in the chapter 2. The alkanes and dichloromethane listed in table 1 were diluted 100

Table 1. List of the compounds tested in the electroantennogram assays and in the wind tunnel

name	compound class	purity	firm	use
pentane	alkanes	99%	Fluka	EAG
hexane		97%	Merck	EAG & WT
heptane		99%	Fluka	EAG & WT
octane		99%	Fluka	EAG & WT
nonane		99%	Fluka	EAG
decane		98%	Fluka	EAG
dichloromethane	halogenic compound	99.9%	Merck	EAG
acetone	ketone	99.8%	Akros	EAG & WT
1-octen-3-ol	alcohol	97%	Merck	WT
POC mixture	alcohol & phenols	Ujváry lab		WT

POC mixture made of the conventional formulation of the 3*n*-propylphenol + 1-octen-3-ol + *p*-cresol 1:4:8 (by weight) mixture (IAEA-TECDOC 2003).
Compounds marked EAG were used for electroantennogram recordings and compounds marked WT were tested in the wind tunnel.

and 1'000 times in hexadecane, and acetone was dissolved in water. The EAG responses measured to hexadecane and water were marginal (less than 10% of the EAG response normalised relative to 1-octen-3-ol, above). Using a glass syringe (Microliter #7001, Hamilton, USA) 1µL of the diluted compound was deposited on a filter paper strip (0.8cm×3cm) placed

inside a 5mL polypropylene syringe (BD Plastipak™, Spain) that served as the stimulus source. For the higher amounts tested, volumes of 0.1µL, 1µL and 10µL of neat products corresponding respectively to 100µg, 1000µg and 10000µg were deposited on the filter paper strip directly inside the 5mL polyethylene syringe using a 1µL glass syringe (Microliter #7001, Hamilton USA) for the 0.1µL and 1µL volumes and a 10µL glass syringe (PN002800, SGE, Australia) for the 10µL volume.

Results

The C_5 to C_{10} alkanes were tested with antennae of two different tsetse fly species and compared with responses to acetone and dichloromethane as a function of dose (Fig. 2&3). The patterns of EAG response obtained with the different products were similar for the two species, in that the antennal receptor cell responses increased exponentially with dose for all the products tested (at more than 2 doses). The EAG responses to the C_5 to C_{10} alkanes provide a hyperbolic curve with highest responses to C_7, C_8 and C_9. Acetone evoked a high EAG response only when 10µL of pure compound (10´000µg) were put on the filter paper strip in the stimulus syringe, revealing higher threshold for acetone than for any of the 6 alkanes tested. The response to dichloromethane was similar to the shorter and the longer alkane products tested.

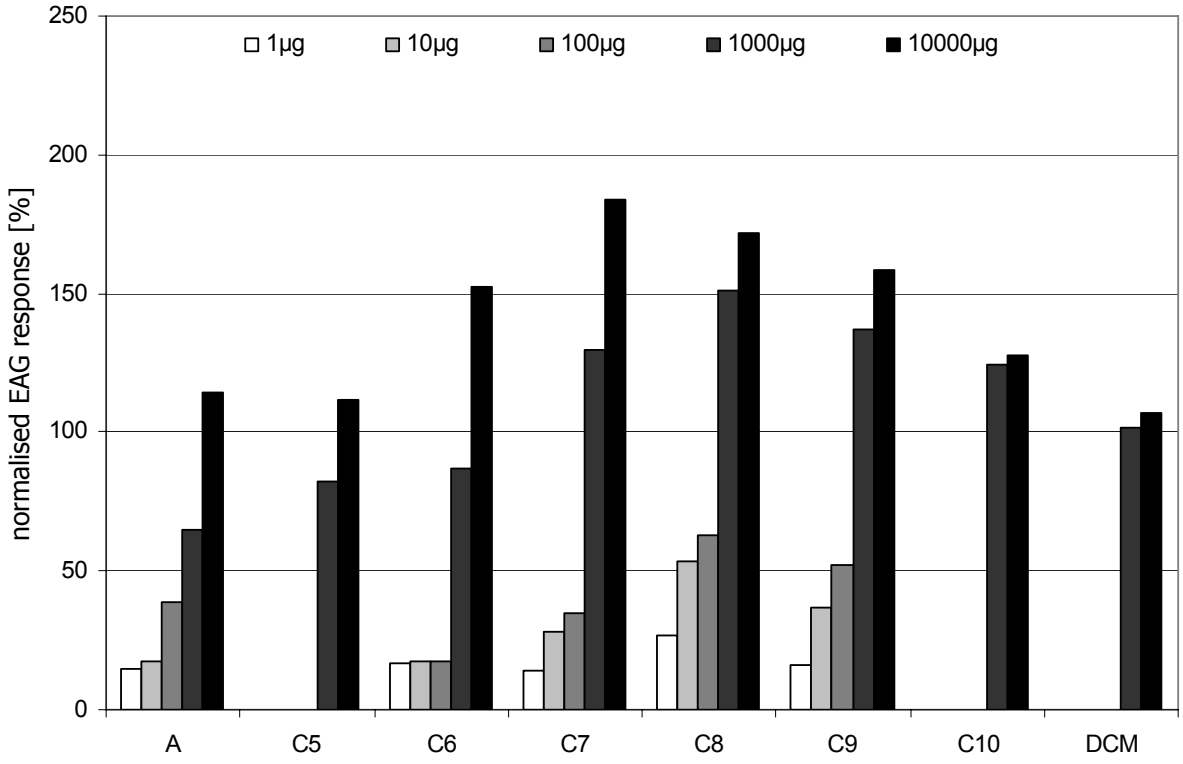


Figure 2. Mean electroantennogramme responses of *G brevipalpis* to acetone, C5 to C10 alkanes and dichloromethane at different concentrations in the air.

The antennal responses of *G. brevipalpis* were recorded to air puffs of the pure compounds acetone (A, n=2,2,4,4,2 antennae), pentane (C5, n=3,2), hexane (C6, n=2,2,4,4,2), heptane (C7, n=2,2,4,4,2), octane (C8, n=2,2,4,4,2), nonane (C9, n=2,2,4,4,2), decane (C10, n=3,2) and dichloromethane (DCM, n=1,1,2) at 5 different doses, ranging from 1µg to 10000µg, put on the filter paper strip in the stimulus syringe. The responses were normalised using an air puff from 100ng of 1-octen-3-ol in a stimulus syringe as reference at the start and end of each recording period.

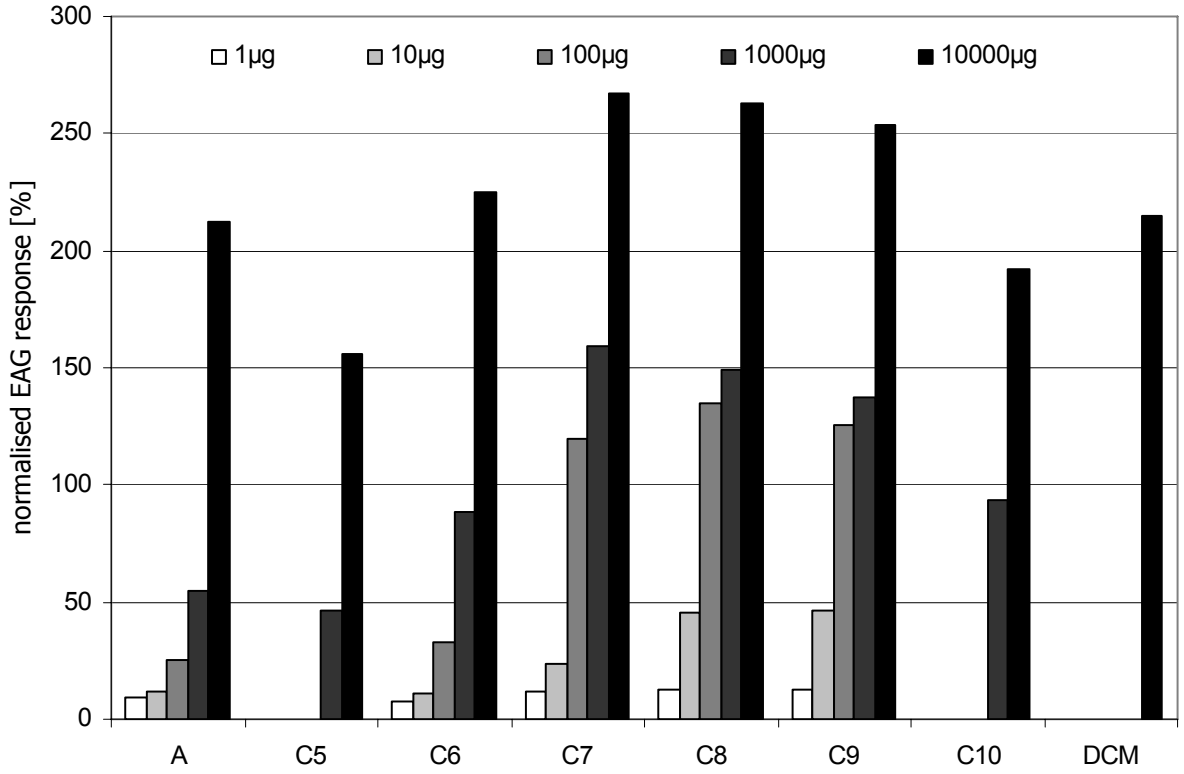


Figure 3. Mean electroantennogramme responses of *G pallidipes* to acetone, C5 to C10 alkanes and dichloromethane at different concentrations in the air.

The antennal responses of *G. pallidipes* were recorded to air puffs at 5 different amounts ranging from 1µg to 10000µg of the pure compounds acetone (A, n=2,2,2,2,2 antennae), pentane (C5, n=3,2), hexane (C6, n=2,2,2,4,2), heptane (C7, n=2,2,2,5,2), octane (C8, n=2,2,2,4,2), nonane (C9, n=2,2,2,4,2), decane (C10, n=3,2) and dichloromethane (DCM, n=2) put on the filter paper strip in the stimulus syringe. The responses were normalised using an air puff from 100ng of 1-octen-3-ol in a stimulus syringe as reference at the start and end of each recording period.

The behavioural responses of *G. brevipalpis* to acetone, hexane, heptane and octane were recorded in the wind tunnel in combination with products known to affect tsetse fly captures in field traps. The release rate of the four test compounds from a 1L gas-wash bottle varied greatly, ranging from more than 15mg/h for hexane and acetone to less than 5mg/h for heptane and octane (Tab. 2).

Table 2. Release rate of the compounds used in the behavioural experiments.

	acetone	hexane	heptane	octane	1-octen-3-ol
release rate [mg/h]	19.2	15.4	4.5	1.3	0.89
mean quantity delivered [µg/L]	2.67	2.14	0.63	0.18	0.12

As for other wind tunnel experiments, the neat product (5mL) was deposited directly in a 1L gas-wash bottle. The mean evaporation rate was calculated by weight losses (n>20) measured over at least 2 consecutive days. For 1-octen-3-ol, a polyethylene sachet (4cm×5.5cm, thickness 150µm) was filled with 1mL of pure compound, sealed and weighed 21 times over 4 consecutive days while hanging in a fume hood with a draught (900m^3/h).

An approximation of the quantity delivered into the test plume in the wind tunnel was then calculated (see *Material & Methods* of *chapter 2* for details).

The known attractive compounds 3*n*-propylphenol, 1-octen-3-ol and *p*-cresol were released into the wind tunnel at an amount close to the amount of octane released (Tab. 1 and see *Material & Methods*).

When acetone, hexane, heptane and octane were tested as behavioural stimulants on their own for *G. brevipalpis*, only acetone elicited behavioural responses significantly different from the negative control (air alone) with 15 of the 40 flies activated, 12 exiting the release cage, 9 making directed flight and 8 reaching the source (Fig. 4). Moreover, some behaviour such as cage exit was significantly higher for acetone than for the alkanes with 12 flies exiting with acetone compared to only 2, 2 and 1, respectively, for hexane, heptane and octane.

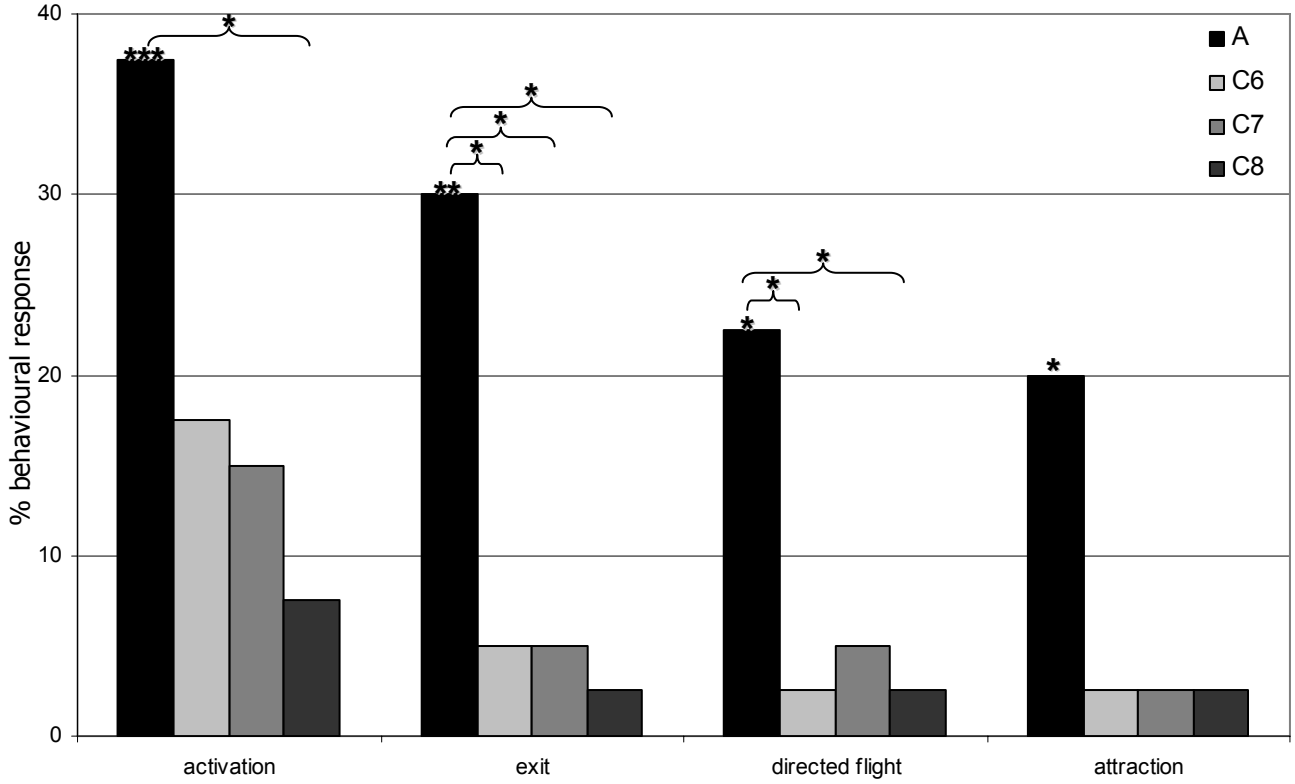


Figure 4. Behavioural responses of *G. brevipalpis* tested for responses to acetone and alkanes in the wind tunnel.

Behavioural criteria recorded were: *activation*, the fly moved within the cage; *exit*, the fly flew out of the cage; *directed flight*, the fly travelled at least 50cm within the plume of odour; *attraction*, the fly made a directed flight to whithin 10cm of the source.

During the 1min test period 0.5L of pure air passed through a 1L gas-wash bottle containing 5mL of acetone (A), hexane (C6), heptane (C7) or octane (C8). The amount of compounds delivered into the plume in the wind-tunnel was estimated as 2.7µg/L for acetone, 2.1µg/L for hexane, 0.6µg/L for heptane and 0.2µg/L for octane. During the negative control (charcoal-filtered air delivered at 0.5L/min) no behavioural responses were recorded.

Each treatment was tested with 20 females and 20 males flies; asterisks indicate that the percentage response was significantly different from that of pure air at $P < 0.05$ (*), 0.01(**) or 0.001 (***) levels of probability using Pearson's Chi-squared test with Yates' continuity correction.

Tests with 1-octen-3-ol alone showed that this molecule only permitted slight activation of *G. brevipalpis* with only 3 flies activated of 15 tested and none showing any other behavioural

responses (Fig. 5). Adding 1-octen-3-ol to either acetone, hexane, heptane or octane increased the behavioural responses to the alkanes, but strongly decreased the different behaviour responses recorded by *G. brevipalpis* to acetone. The same effect on activation was observed

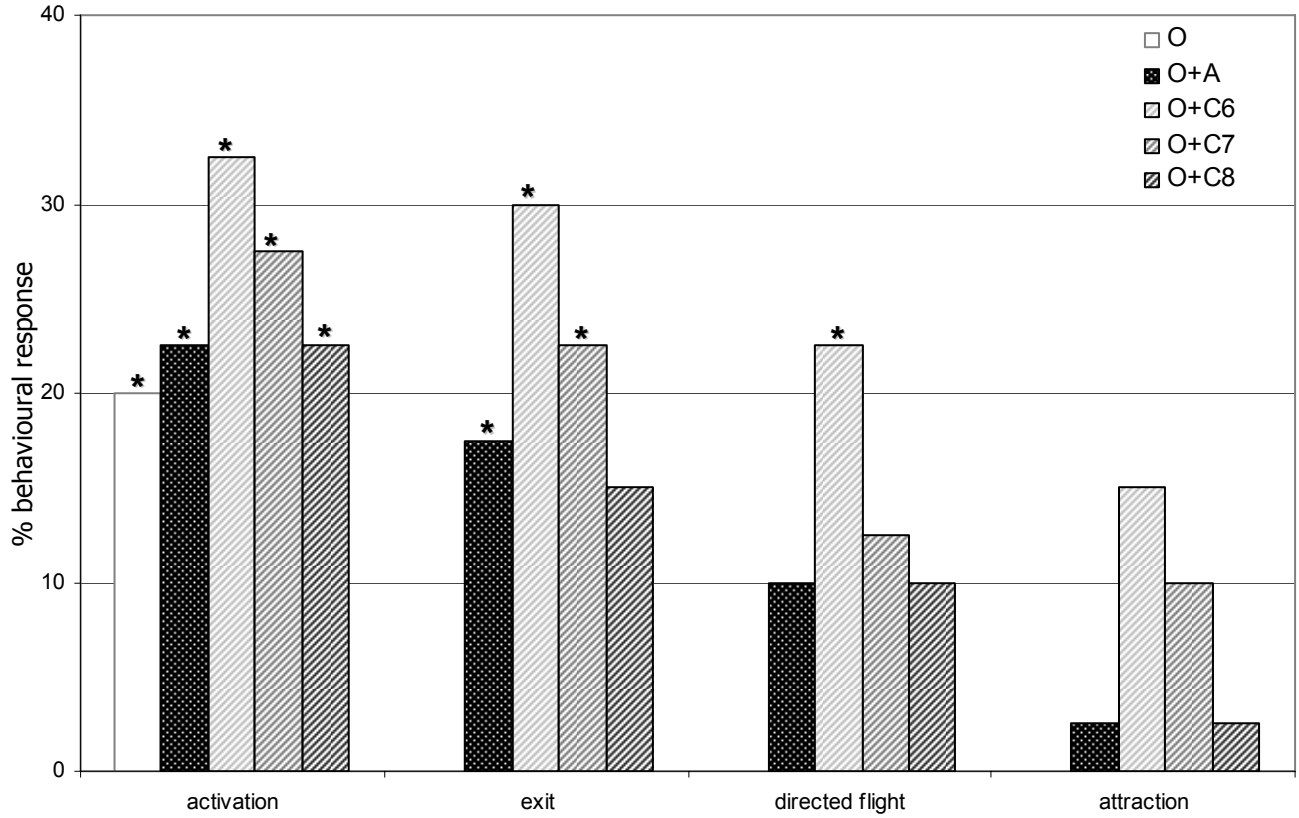


Figure 5. Behavioural responses of *G. brevipalpis* to acetone, hexane, heptane or octane plus 1-octen-3-ol and delivered into the odour plume in the wind tunnel.

The behavioural criteria recorded, the release rates of acetone and alkanes, the negative control and the statistics were already described in the legend to *Figure 4*.
During the 1min test period 0.5L of air passed through a 1L gas-wash bottle containing 5mL of acetone (A), hexane (C6), heptane (C7) or octane (C8) and was mixed with 5mL of air passing through a 1L gas-wash bottle containing a polyethylene sachet (4cm×5.5cm, wall thickness 150µm) containing 1mL of 1-octen-3-ol. Only 15 flies were tested with octenol (O).

with 1-octen-3-ol admixed with either acetone or the alkanes, with 9 to 13 flies over the 40 tested showing activation in the cage. This level of activation is not different to that observed with 1-octen-3-ol alone. Cage exit only increased significantly for the mixtures of hexane, heptane or acetone added to 1-octen-3-ol. In the case of acetone with 1-octen-3-ol added only 9 flies were activated of which only 1 reached the source, whereas 8 flies of 40 reached the source with acetone alone. The best response was obtained with hexane plus 1-octen-3-ol, inducing significantly higher behavioural responses in the behavioural steps up to directed flight: of the 40 flies tested, 13 were activated, 12 exited the release cage and 9 engaged a directed flight with 6 reaching the source. Whereas the release rate of octane was 10 times lower than that of acetone (Tab. 1), responses by *G. brevipalpis* to 1-octen-3-ol plus acetone or to 1-octen-3-ol plus octane were equivalent for all behavioural criteria recorded i.e. similar numbers of flies responding at each behavioural step of the 40 flies tested.

The addition to acetone or the alkanes of the ternary mixture of 3*n*-propylphenol, 1-octen-3-ol and *p*-cresol (POC) released from the polyethylene sachet increased the behavioural response under all the criteria recorded for *G. brevipalpis* (Fig. 6). POC alone was not very effective inducing 1 exit from the release cage of 40 flies tested. Adding acetone (A) to the ternary POC mixture (producing POCA) increased activity with 14 flies activated by this blend exiting the cage and 9 of them reaching the source. Addition of heptane to POC mixture induced levels of fly behaviour similar to the POCA mixture with 15 flies activated, 7 making a directed flight and 5 reaching the source. Hexane added to POC still induced a significant effect on activation with 9 flies activated but even though the levels of behaviour recorded for the other criteria were higher than for POC alone, they were not significantly different from the blank control. Furthermore, this mixture was less effective than the hexane plus 1-octen-3-ol mixture (compare Figs. 5 & 6). Octane induced no significant effects on any of the behaviours of *G. brevipalpis* when added to POC, but the behavioural effects of this mixture were higher under all the criteria recorded compared to POC alone.

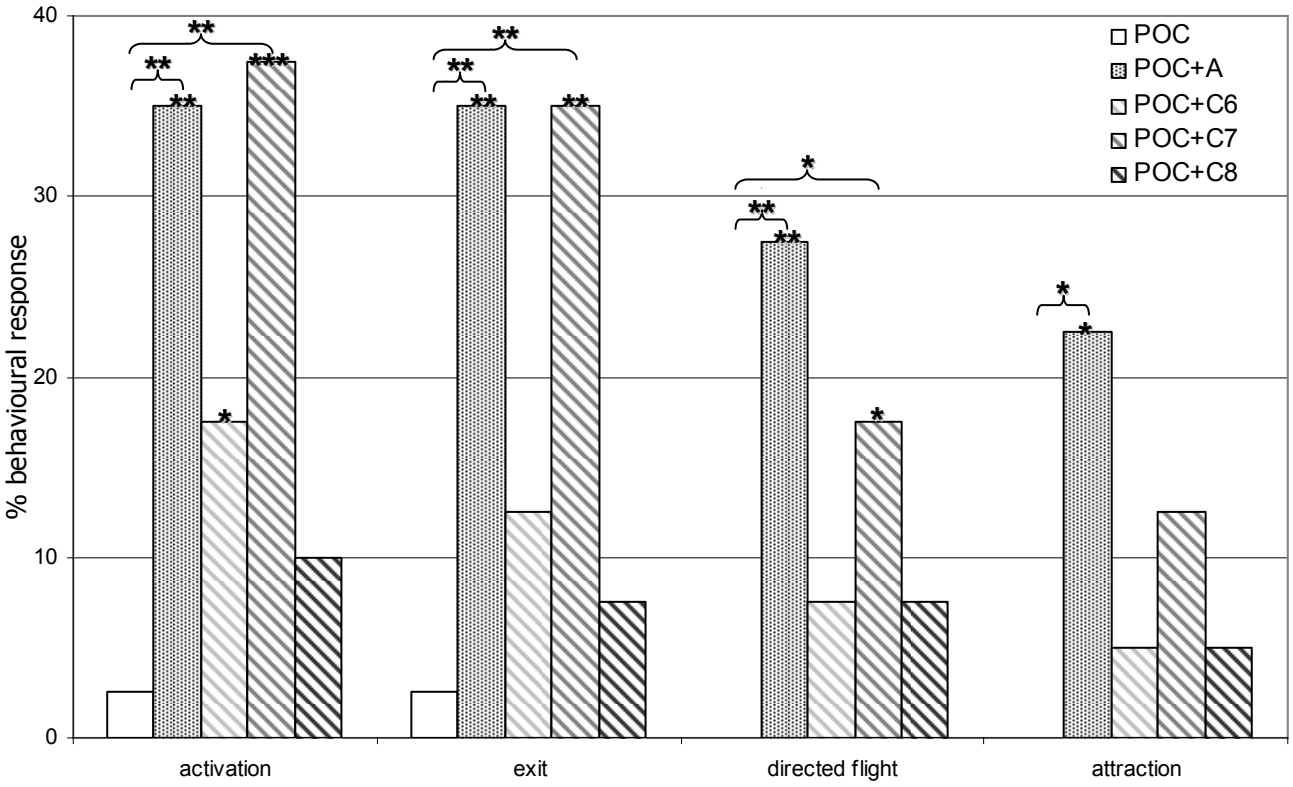


Figure 6. Behavioural responses of *G. brevipalpis* in the wind tunnel to acetone, hexane, heptane and octane added to the tsetse fly attractants 3*n*-propylphenol, 1-octen-3-ol and *p*-cresol as released from a polyethylene sachet.

The behavioural criteria recorded, the release rates of acetone and alkanes, the negative control and the statistics were already described in the legend to *Figure 4*.
During the 1min test period 0.5L of air passed through a 1L gas-wash bottle containing 5mL of acetone (A), hexane (C6), heptane (C7) or octane (C8) mixed with 5mL of air passing through a 1L gas-wash bottle with a polyethylene sachet (4cm×5.5cm, thickness 150µm) containing a mixture of 3*n*-propylphenol, 1-octen-3-ol and *p*-cresol at a ratio 1:4:8, respectively.

The improvement of the behavioural responses of *G. brevipalpis* through the addition of either acetone, hexane, heptane or octane to 1-octen-3-ol plus the 2 phenols was relatively small and most of the time not even significant (Tab. 3). For example with the most significant treatment of the binary mixtures in terms of directed flight 9 flies respondied to hexane plus 1-octen-3-ol whereas for the ternary mixture of POC mixed with hexane the number of directed flight were just 3 of 40 flies tested. The levels of activity recorded for the different behavioural criteria between treatments were too small for valid comparisons.

Table 3. Summary of the behavioural responses recorded in the wind tunnel for *G. brevipalpis* in response to acetone, hexane, heptane or octane alone and to these 4 products tested with 1-octen-3-ol or the ternary mixture of 3n-propylphenol, p-cresol and 1-octen-3-ol used in a polyethylene sachet.

	activation		exit		directed flight		attraction	
A	37.5%	a	30.0%	abc	22.5%	abcd	20.0%	abcd
O+A	22.5%	abcd	17.5%	abcde	10.0%	bcdef	2.5%	ef
POC+A	35.0%	ab	35.0%	ab	27.5%	abc	22.5%	abcd
C6	17.5%	abcde	5.0%	def	2.5%	ef	2.5%	ef
O+C6	32.5%	ab	30.0%	abc	22.5%	abcd	15.0%	abdef
POC+C6	17.5%	abcde	12.5%	abcdef	7.5%	cdef	5.0%	def
C7	15.0%	abdef	5.0%	def	5.0%	def	2.5%	ef
O+C7	27.5%	abc	22.5%	abcd	12.5%	abcdef	10.0%	bcdef
POC+C7	37.5%	a	35.0%	ab	17.5%	abcde	12.5%	abdef
C8	7.5%	cdef	2.5%	ef	2.5%	ef	2.5%	ef
O+C8	22.5%	abcd	15.0%	abdef	10.0%	bcdef	2.5%	ef
POC+C8	10.0%	bcdef	7.5%	cdef	7.5%	cdef	5.0%	ef
POC	2.5%	ef	2.5%	ef	0.0%	f	0.0%	f
pure air	0.0%	f	0.0%	f	0.0%	f	0.0%	f

The behavioural criteria recorded, the release rates of acetone and alkanes and the negative control were already described in the legend to *Figure 4*.
Treatments were tested with 20 females and 20 males. In the table behavioural activity levels followed by the same letter are not significantly different (P>0.05) using Pearson's Chi-squared test with Yates' continuity correction.

The changes in the behavioural responses of *G. brevipalpis* elicited by heptane varied depending on what was added to it (Fig. 7). For all the 4 behavioural criteria recorded, the behavioural response to heptane was improved by addition of 1-octen-3-ol, an effect that was even more visible when 3*n*-propylphenol and *p*-cresol were added to heptane with 1-octen-3-ol. Whereas only 2 flies of 40 flew from the cage when stimulated by heptane on its own, addition of 1-octen-3-ol to it caused 9 flies of 40 to exit the release cage, and with 3*n*-propyl phenol and *p*-cresol added to this binary mixture 14 flies of 40 flew from the cage, significantly better than result with heptane on its own.

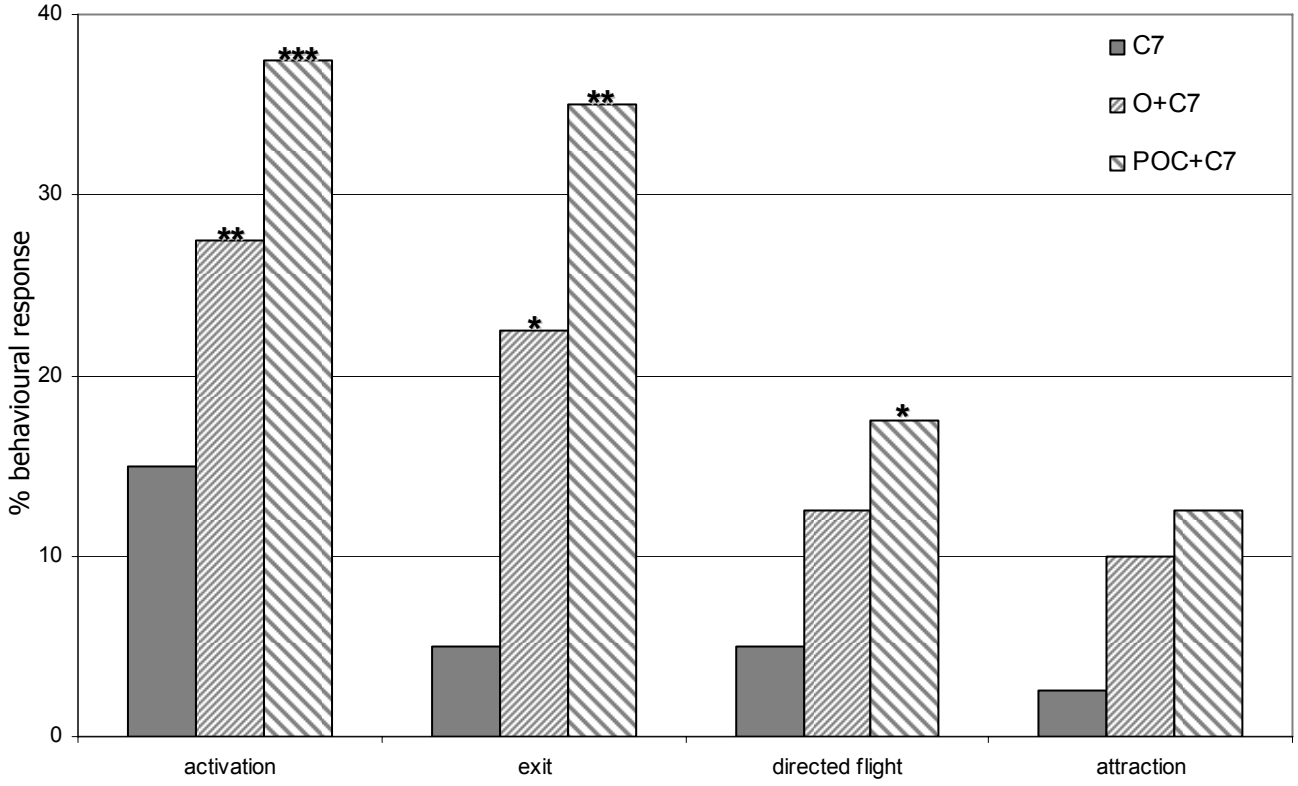


Figure 7. Behavioural responses of *G. brevipalpis* in the wind tunnel by heptane, heptane plus 1-octen-3-ol and heptane plus 1-octen-3-ol, 3*n*-propylphenol and *p*-cresol.

The behavioural criteria recorded, the release rates of acetone and alkanes, the negative control and the statistics were already described in the legend to *Figure 4*.

Discussion

That alkanes should attract insects is not unexpected. Various studies already report effects on insects of these compounds when used as a solvent (Healy & Jepson, 1988; Puri *et al.*, 2006; Kröber, *unpublished data*) and, like acetone, alkanes occur in breath as reported by many authors (Tab. 4). As we have shown in the previous chapter, breath contains a very rich profile of products and human breath is attractive to *G. brevipalpis* (Chapter 3 of this thesis).

Table 4: Partial list of alkanes found by diverse authors in breath of human and cattle.

compounds	human breath	cow breath
pentane	BCDE	
trimethylpentane	BCD	
hexane	ABC	a
3-methylhexane	BCD	
methyl cyclohexane	ABCD	
heptane	ABCDE	a
dimethyl heptane	ABD	
octane	ADE	a
nonane	AD	a
decane	ADE	a
dodecane	AD	

References **A**: Krotoszynski *et al.* 1977, **B**: Phillips *et al.* 1992, **C**: Wahl *et al.* 1996, **D**: Phillips *et al.* 1999, **E**: Poli *et al.* 2005, **a**: Spinhirne *et al.* 2004.

Antennae of *G. pallidipes* and *G. brevipalpis* belonging to the *morsitans* and the *fusca* groups of tsetse flies are able to perceive dichloromethane and alkanes such as hexane, heptane and octane, as well as acetone. Similar EAG responses were recorded in *Cx. quinquefasciatus* in response to stimulation with hexane (Puri, Mendki et al. 2006). As for acetone, the sensory threshold of the antennal sensory receptor cells for C_5 to C_{10} alkanes is very high in tsetse flies. At such high doses the receptor cell responses are less specific and for example, dichloromethane may mimic the structure of CO_2 (Jewett and Bjostad 1996). This might explain the similarity in EAG depolarisations evoked by heptane, octane and nonane at the highest doses tested where similar EAG responses were observed for *G. pallidipes* and *G. brevipalpis*. Even though other breath components tested in chapter 3 show a lower threshold than the alkanes or acetone, the olfactory receptor cells of *G. pallidipes* and *G. brevipalpis* do not seem to saturate with the high doses of alkanes delivered to the antennae.

Whereas hexane, heptane, octane or 1-octen-3-ol presented alone just slightly augmented activation in *G. brevipalpis*, when mixed together activation was increased with binary mixtures of any of these alkanes plus 1-octen-3-ol, suggesting a synergistic interaction between these compounds in the activation of tsetse flies. Such synergistic effects were already observed in field experiments between CO_2, acetone and 1-octen-3-ol (Vale and Hall 1985). Hexane released

at 15.4mg/h added to 1-octen-3-ol increased all the behavioural elements recorded for *G. brevipalpis*, but adding 3*n*-propyl phenol and *p*-cresol had a bad effect on this mixture. The mixture of heptane plus 1-octen-3-ol increased the levels of the behavioural elements recorded for *G. brevipalpis* compared to heptane alone, and adding the two phenols to this binary mixture permitted to augment the levels of behaviours recorded. In fact, heptane, released at 4.5mg/h, was the only alkane tested for which the behavioural responses of *G. brevipalpis* were improved by the addition of other compounds. The poor behavioural results obtained with octane, even though this product evoked strong EAG responses, may be explained by the dose employed. The release rate of octane at 1.3mg/h was very low compared to acetone (≈19.2mg/h) or hexane (≈15.4mg/h), but nearly equivalent to the release rates of the known chemostimuli 1-octen-3-ol (at ≈0.9mg/h when presented alone and ≈0.5mg/h in the mixture with the two phenols), 3*n*-propylphenol (≈0.2mg/h) and *p*-cresol (≈1.5mg/h) (Tab. 1; FAO manual, 1992). A similar phenomen was observed by Knols *et al*. (1997) with high concentrations of carboxylic acid mixtures decreasing the efficacy of approaches of *Anopheles gambiae*.

Acetone, at a release rate of 19.2mg/h, attracted *G. brevipalpis* on its own in our wind tunnel experiments. But the addition of 1-octen-3-ol to this ketone decreased its attractive effect as flies exiting the release cage but did not engage in directed flight. In our system, the release rate of 1-octen-3-ol (≈0.9mg/h) was as recommended for traps (FAO 1992), whereas Kappmeier & Nevill (1999) used successfully a much higher release rate (2.3-9.1mg/h) to catch *G. brevipalpis* in the field. As identical conditions can occur near the traps in the field, we conclude that the low behavioural response of *G. brevipalpis* to the mixture of 1-octen-3-ol plus acetone was due to the suboptimal ratio of the mixture. When acetone was released with the volatiles from the polyethylene sachet containing the two phenols in addition to 1-octen-3-ol, the number of flies completing the four behavioural criteria reached a level slightly higher than for stimulation with acetone on its own. 3*n*-Propylphenol and *p*-cresol probably masked the suboptimal effect of 1-octen-3-ol in the mixture with acetone.

Even though the increase in response with the chemostimuli from the sachet plus heptane was not higher than the response for sachet-released products plus acetone, heptane could provide a reasonable alternative to acetone in an effort to reduce the trap maintenance as its volatility is much lower than that of acetone (vapour pressure at 30°C equal to 283mmHg for acetone and 58mmHg for hexane). Because of its high volatility, acetone may only be acting as a substitute for CO_2 (Torr 1990) as a close range attractant. As the heptane release rate is intermediate between acetone and the compounds coming from the polyethylen sachet and since the final approach of tsetse to hosts is mainly visual (Torr 1989), heptane may serve to increase the range of attraction by interacting with the three other compounds.

Not many field tests have been carried out on tsetse flies using alkanes but in Zimbabwe, Vale (1980) obtained an increase in *morsitans* spp. capture by baiting traps with hexane released at 20g/h from an opening bottle and CO_2 (2.5L/min), but the increase of 1.7 fold was not significant compared to trap catches with CO_2 alone. We can suggest that the high volatility of compounds such as acetone serves to increase the attractive range of compounds.

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General discussion

Research on tsetse fly sensory ecology permits to extend our knowledge of this important group of disease vectors. Beyond their well developed visual system for guiding their final approach to a host, tsetse flies use olfactory stimuli to find a blood meal from a distance (Bursell 1984). As demonstrated in *chapter 1* of this thesis, a precise analysis of tsetse flight captured in 3D permitted to characterize flight behaviours in the presence or absence of a visual target. In the field, the design, colour and location of visual traps has been studied in detail, and nowadays provide good baits for tsetse flies. The addition of odour to these traps increases their range of attraction and permits to reduce trap density in the field without reducing the likelyhood of catching flies (Williams, Dransfield et al. 1992). In the same manner, early studies on tsetse fly physiology similar to those placed in the annexes to this thesis have improved our understanding on the tsetse fly biology. In addition, the analysis of tsetse fly flights to different metabolites in a controlled environment such as the wind tunnel used here can help in the development of attractants for field use.

The benefit of baiting traps with odour attractants varies between tsetse species. Research to date has concentrated mainly on savannah tsetse fly species and on a few products identified in host odours that attract these species. Even if among the several hundreds volatiles emanating from hosts only a small subset serve as cues that guides haematophagous arthropods, the olfactory perception by tsetse flies of volatile host metabolites evidently needs to be more extensively studied. To achieve this, rich substrates such as rumen fluid of cattle and vertebrate breath were considered here as sources of material to investigate the sensory adaptations of tsetse flies from the three subgenera. Carboxylic acids, the predominant metabolites in the rumen fluid of cattle, are perceived by tsetse flies from the three subgenera (*Chapter 2* of this thesis). A more detailed study on *G. brevipalpis* demonstrated a low threshold for cyclo hexane carboxylic acid. However, we were not able to show an increase in attraction due to these carboxylic acids in our wind tunnel experiments, but demonstrated, contrary to what was concluded in previous field studies made with disproportionally high doses (Vale, 1980; Torr *et al.*, 1996), that carboxylic

acids such as pentanoic and hexanoic acids do not elicit repulsive behaviours in tsetse flies. This matches with data of Knols *et al.* (1997) who showed that a mixture of carboxylic acids that attracts mosquitoes at low doses, serves to repel them at higher doses. Moreover, butanoic, pentanoic, hexanoic and isobutanoic acids presented in proportions similar to that found in the rumen and dissolved in DCM attracted *G. pallidipes*. In any case, the carboxylic acids produced in the rumen are used as a source of energy by the herbivores and consequently only a small amount is "accidentally" released via breath during exhalation of CO_2 and CH_4 (Mackie, Stroot et al. 1998). Therefore, depending of the host, breath can contain a large mixture of metabolites, with a "small core" of commonly occurring volatile organic compounds coming from aerobic and anaerobic pathways that can affect the behaviours of a range of haematophagous arthropods. In *Chapter 3* of this thesis, breath metabolites from different chemical classes were tested on the olfactory system of different tsetse fly species using the electroantennogram, and in *Chapter 4*, the behavioural responses of *G. brevipalpis* to one of these classes, the alkanes, were recorded. Among these breath metabolites most of them were already known as having an influence on haematophagous arthropod behaviour, and such metabolites can also originate from various body parts of the hosts. For instance, the aliphatic aldehydes that are perceived by tsetse flies (*chapter 3*) are present in breath (Phillips *et al.*, 1999; Spinhirne *et al.*, 2004) and rumen fluid (Jeanbourquin 2005) as well as in skin emanations from different vertebrates (Burger *et al.*, 1999a; Gikonyo *et al.*, 2002; Wood & Weldon, 2002; Curran *et al.*, 2005) and serve to modify the behaviours of different blood sucking arthropods such as mosquitoes (Du and Millar 1999), triatomine bugs (Guerenstein & Guerin, 2001) and ticks (McMahon and Guerin 2002). Plant compounds such as terpens, which can be found in breath after passing untransformed through the digestive tracks of ruminants, are perceived by the strictly haematophagous tsetse flies, and as suggested by Syed & Guerin (2004) can be used parsimoniously depending of the needs of the insect to find a host or shelter, i.e. dependent on its physiological state. In this thesis, we observed that the addition of heptane to *3n*-propyl phenol, 1-octen-3-ol and *p*-cresol permits attraction of *G. brevipalpis*, and could provide a reasonable alternative to acetone.

The data presented in this thesis demonstrate that tsetse flies are able to sense and respond behaviourally to a much wider range of products than 3*n*-propyl phenol, 1-octen-3-ol, *p*-cresol and acetone currently used to capture tsetse flies in the field. Moreover, it is interesting to point out that some compounds shown to be of importance for tsetse flies here have common origins or biosynthetic pathways. For example, linoleic acid is one of the five most frequently occurring fatty acids in plant tissues and is consequently readily found in the digestive tracks of herbivores. It plays an important role in membrane building and functioning of all cells, and it is implicated in the biosynthes of various products. As indicated in different chapters of this thesis linoleic acid can be transformed into different compounds perceived by tsetse flies such as 1-octen-3-ol, 3-octen-2-one, pentane and hexanoic acid (Fig 1). In addition, primary alcohols, aldehydes and carboxylic acids can occur both as products of and substrates for oxidation / reduction reactions (Fig 2). Degradation of amino acids permits the formation of straight- or branched-chain fatty acids resulting from the deamination of simple amino acids such alanine, glycine, leucine or valine. Aromatic amino acids such tryptophan and tyrosine can be transformed, into indoles and phenols that are also sensed by tsetse flies. One can speculate that the electroantennogram responses recorded for benzothiazole and cyclo hexane carboxylic acid might be linked to the atomic configuration similarity between these molecules and, respectively, indole and *p*-cresol (Fig. 3). Similar mimicry has already been suggested by Jewett & Bjostad (1996) between dichloromethane and CO_2 affecting diabroticite larvae, and by Saini (1989) between different 1-octen-3-ol analogues for *G. m. morsitans*. Nevertheless, the sulfur atom of benzothiazole might in itself play a role in tsetse fly sensory responses since dimethyl trisulfide also elicited electroantennogram responses and these sulfur products can also arise from the degradation of the amino acids cystein and methionine.

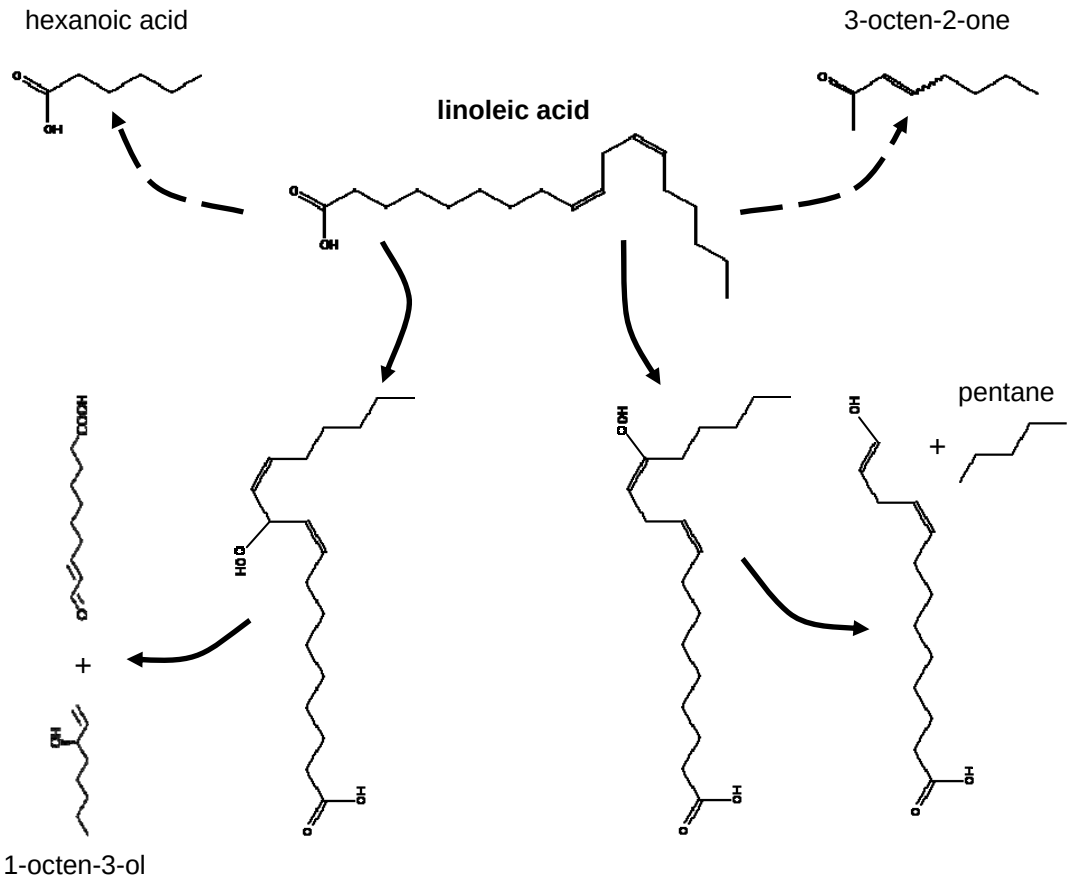

Figure 1. Different pathways of linoleic acid degradation

Dashed arrows represent incomplete pathways for the formation of hexanoic acid and 3-octen-2-one.

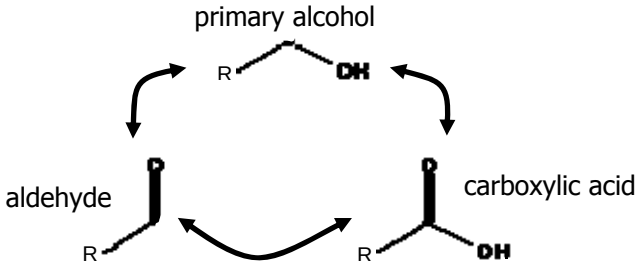

Figure 2. Possible interchanges between compound families through oxidation/reduction reactions

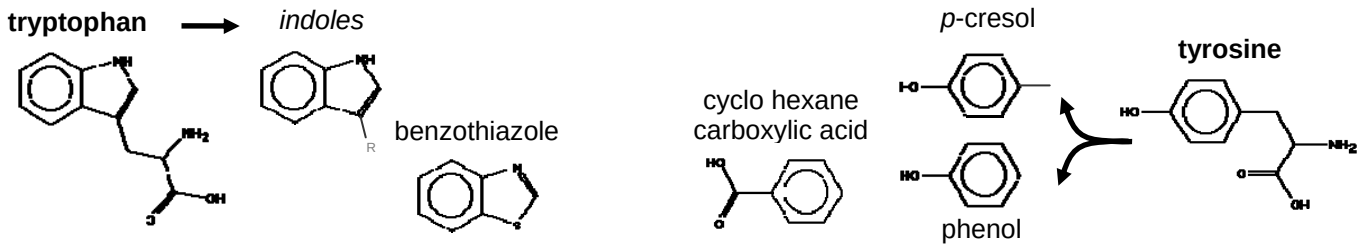

Figure 3. Structural similarities between compounds formed through amino acid degradation.

Benzothiazole and indoles are formed from tryptophan, cyclo hexane carboxylic acid and phenols are formed from tyrosine.

The attractiveness of haematophagous arthropods to metabolites arising from the degradation of biological substrates suggests that these organisms use such signals to inform themselves about the presence of an available source of energy. Moreover, host recognition from a distance by haematophagous arthropods seems to occur from sensitivity to combinations of products of different functionality arising from different metabolic pathways of their hosts. The attractants used today for tsetse flies, namely acetone, 1-octen-3-ol and phenols come from different biosynthetic origins but are all polar compounds. The addition of apolar compounds such as alkanes could permit improvement of the behavioural responses by stimulating another channel in the tsetse fly antennal lobe. Avoiding redundancy between compounds in an attractive mixture can allow an increase in the behavioural response contrary to inclusion of compounds in a mixture that activate the same olfactory receptor cell pathway to the brain and that may be interpreted as a too high dose leading to repulsion. Moreover, when ligand-specific pathways are activated different glomeruli are stimulated. We can imagine that in addition to excitatory interactions, suggested by Olsen *et al.* (2007), inhibitory feedback mediated by local interneurones between glomeruli may also exit, as mentioned by Laurent (2002). The risk of an overdose in any one channel is less likely and consequently raises the sensory threshold at which repulsion may occur.

The electroantennogram has shown itself here to provide an appropriate method to investigate and test the general sensitivity of tsetse flies to olfactory stimuli. Conducting behavioural experiments under the controlled conditions of a wind-tunnel is extremely useful as the assessment of potential attractants through trapping experiments in the field is time consuming and subject to variables such as trap sitting and design, fly population levels, weather and seasonality (Turner 1971). Moreover, the use of modern 3D flight tracking methods are very suitable for recording the behaviours of these very fast flying insects, and particularly for the analysis of qualitative and quantitative aspects of behaviours elicited by different test substances. However, compounds tested in such an artificial and restricted environment must be tested

subsequently in the field before any valid conclusions can be drawn regarding the role of such products may play in tsetse fly sensory ecology.

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Annexes

ANNEX A - Emergence of *Glossina* spp. in the laboratory

Adult tsetse flies emerge after a pupation period that varies according to species and temperature (Fig. 1). Sublethal high temperatures increases the rate of metabolism and thus shortens the pupal duration (Leak 1999).

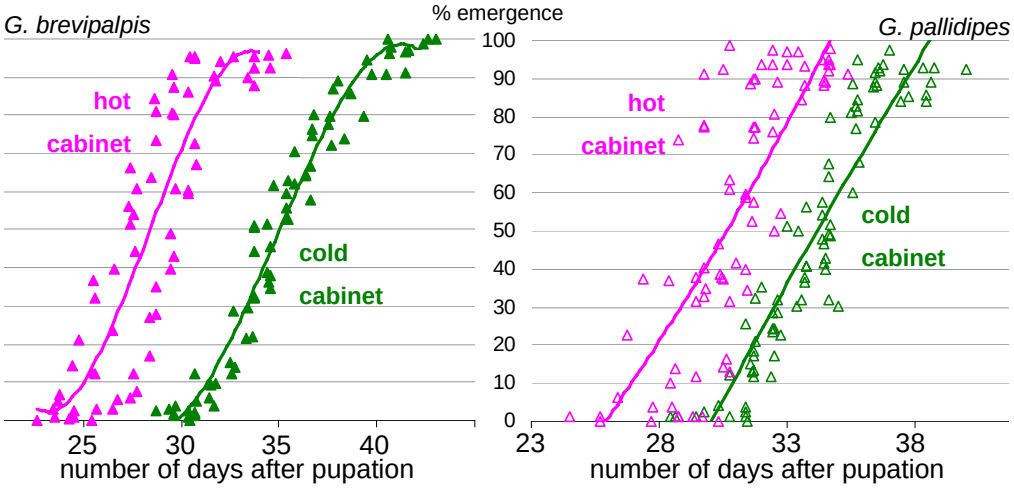

Figure 1. Rate of emergence of *G. brevipalpis* and *G. pallidipes* as a function of temperature.

In this study, tsetse fly pupae (n=4082 for *G. brevipalpis* and n=3270 for *G. pallidipes*) were placed inside two climatic chambers with 100% of humidity and constant 28°C (*hot cabinet*) and an 8h day at 26°C and 16h night at 22°C (*cold cabinet*). 8 replicates were made with *G. brevipalpis* (filled symbols) and 16 replicates were made with *G. pallidipes* (open symbols).

The ratio of the sexes at emergence was close to unity for *G. brevipalpis* and *G. pallidipes*, but there were differences in pupal duration between the sexes with females emerging slightly earlier than males (Birkenmeyer and Dame 1975). Similar results were observed for *G. brevipapis* in this study with pupae held at 2 different conditions (Fig. 2).

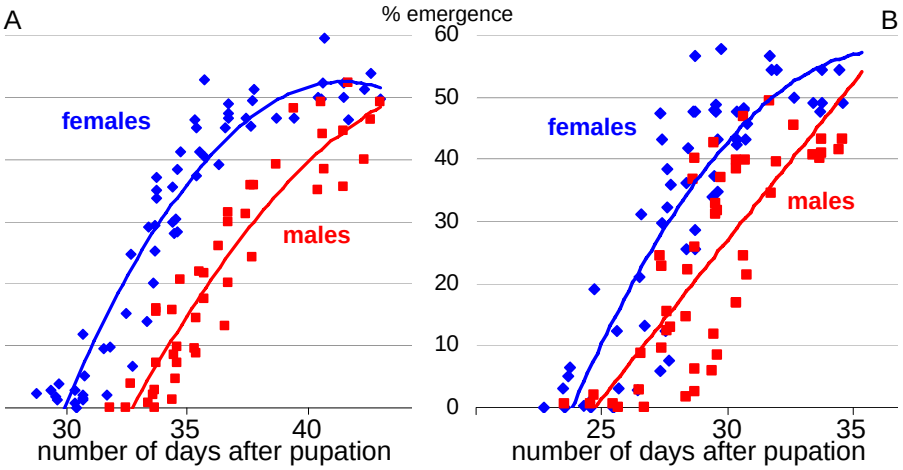

Figure 2. Rate of emergence of *G. brevipalpis* female and male linked to time.

Tsetse flies pupae (n=4082 for *G. brevipalpis*) were placed inside two climate chambers at 100% humidity and an 8h day at 26°C - 16h night at 22°C (A) and the other with a constant 28°C (B). Flies were sexed just after emergence and 8 replicates were made.

The periodicity of eclosion is temperature dependent rather than daylight dependent (Zdárek and Denlinger 1993), and so seasonal variations in climate modify the number of days of the pupal period (Rogers 1990). In nature, a cold season can cause a delayed emergence and unhatched pupariae accumulate in the ground. During this period the survival of the pupae depends on its energetic reserve and adults may not emerge when the development is not completed due to exhaustion of the fat reserves of the pupae (Rajagopal and Bursell 1965; Leak 1999). Similar findings were recorded in the laboratory during this study (Fig. 3)

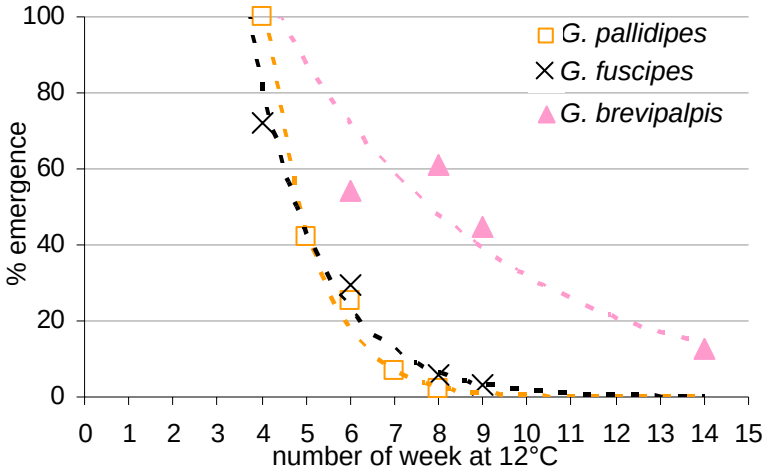

Figure 3. Relation between time spent at 12°C and the rate of emergence of 3 different tsetse fly species.

Tsetse fly pupae (n=4200 for *G. pallidipes*, n=1200 for *G. fuscipes* and n=1500 for *G. brevipalpis*) were stored in an incubator at 100% RH, 12°C. After a variable number of weeks (abscissa) they were placed in a climatic chamber (100% RH, 8h day at 26°C and 16h night at 22°C) for emergence.

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ANNEX B - *G. pallidipes* spontaneous activity in the laboratory

Even though there are some interspecific differences in the diurnal activity pattern of *Glossina* spp. (Gibson and Torr 1999), most species demonstrate a U-shaped spontaneous activity pattern with 2 peaks, one at dawn and one at dusk. This activity is correlated with the field conditions: riverine species of the palpalis group are strongly diurnal, savannah species of the morsitans group are diurnal but with less midday activity, and forest species of the fusca group are strongly crepuscular (Gibson and Torr 1999). Activity of the savannah species, *G. pallidipes* was recorded during this study using a webcam (PCVC740K, ToUcam, Phillips) linked to a software package CamTud3 (University of Neuchâtel).

Simultaneous recording of 10 *G. pallidipes* in a cage showed that a high proportion of them moved in the cage during the photophase (Fig. 1A), certainly due to disturbance between flies (Warnes 1989). However 2 peaks of activity were visible, with less flies activated around noon. Recordings of flies alone in the cage showed less activity, but 2 peaks of activity were detectable during the morning and the evening (Fig. 1B). These findings are in close agreement with previous observations, and particularly with the data of Brady (1974) on an other tsetse fly species of the morsitans group, *G. morsitans*.

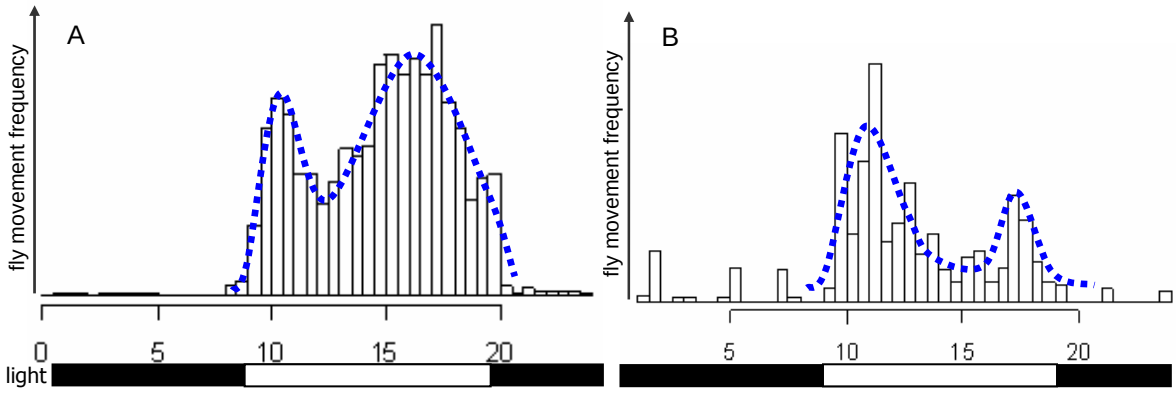

Figure 1. Frequency of movements by *G. pallidipes* over the day.

A Movements of 10 *G.pallidipes* female were recorded in a cubic cage with 35cm sides over 5 consecutive days. B Movements of 1 *G. pallidipes* male were recorded in a cubic cage with 35cm sides over 6 consecutive days.
The black and white bars below the graphs indicate the photophase (white bars, 10h) and scotophase (black bars, 14h).

Bursell (1961) divided the inter meal sessions into 4 distinct phases with the insect is inactive (after blood meal), the insect approaches and follows a moving object, the insect is strongly attracted by stationary baits and the insect is strongly attracted to moving object. Tsetse flies are motivated by a blood meal during the 2 last phases namely the early stage of hunger and the hunger phase when its lipid reserve is depleted and acquisition of food is increasingly urgent, but Langley & Wall (1990) claimed that it is impossible to distinguish between phases. Using a similar system as described above, cages with 10 newly emerged and starved *G. pallidipes* were filmed and the cumulative number of movements quantified over 5 and 7 days of recording. Total activity increased until days 4 and 5 before decreasing, partly due to fly death (Fig. 2). The increase in activity over the days is in accordance with the findings of Brady (1975) who showed that the spontaneous activity of *G. morsitans* increases during the 5 first days of starvation. Similarly, van der Goes van Naters *et al.* (1998) found that the number of biting bouts on a warm paper disc and walking time increases over 4 successive days of starvation in *G. m. morsitans* and *G. f. fuscipes*.

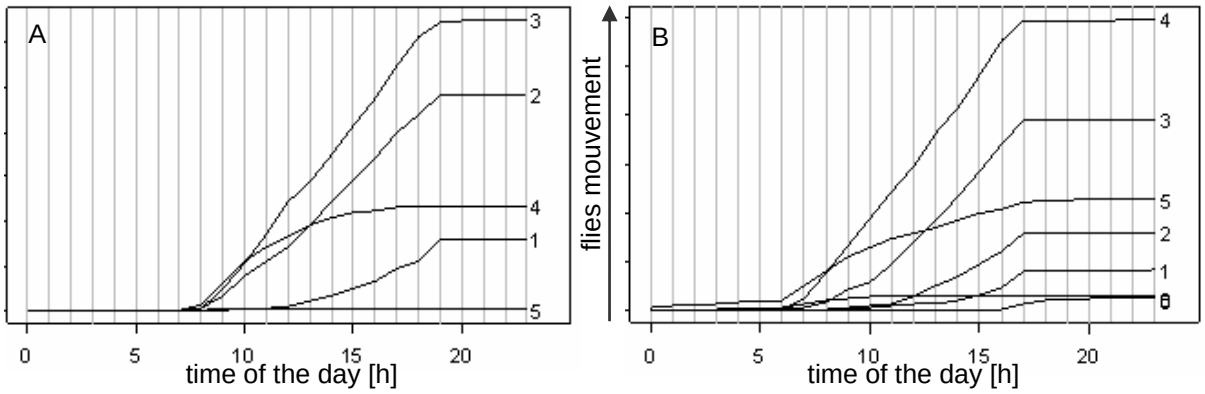

Figure 2. Measurements of *G. pallidipes* activity over several days.

Cubic cages with 35cm sides contained 10 *G. pallidipes* males (A) and females (B). Flies emerged at day 0 and the number of movements they made was recorded over 5 days (A) and 7 days (B). Numbers at the end of the curves correspond to the day number and the light:dark cycle is as figure 1.

Studies on spontaneous activity and on metabolism (*Annex C*) of tsetse flies permitted to plan the schedule for behavioural experiments in the wind tunnel.

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ANNEX C - Measurements of flight muscle and energetic reserves of *G. pallidipes* in the laboratory

The aim of this work was to quantify the flight muscles and the energetic reserves of tsetse flies in our insectarium. These physiological measurements were made on *G. pallidipes* by applying the protocol of Terblanche et al. (2004). Alive tsetse flies were weighed (AE100 balance, Mettler-Toledo, Switzerland) at emergence and before and after each blood meal. They were then sacrificed using CO_2 as anesthetic, head, thorax and abdomens were removed, and measurements were made on the thorax and abdomens. Fresh (just after killing), dry (dried in an oven at 60°C to a constant mass (<72h)) and lipid free (gently perforated, washed 3 times for 24h in solvent-based lipid extractions with chloroform:methanol at a ratio 2:1 and then dried in an oven at 60°C to a constant mass (<48h)) were then weighed. The length of the thorax was measured with vernier caliper (Series 531; Mitutoyo, Japan) along the lateral face of the thorax from beneath the fold of the scutellum to the front face of the pronotum (Fig. 1A). The wing was measured from the distal base of the fold of the alula to the tip of the wing with the vernier caliper (Fig. 1B). However, as stated by Hargrove (1975), there is a large variability between individuals.

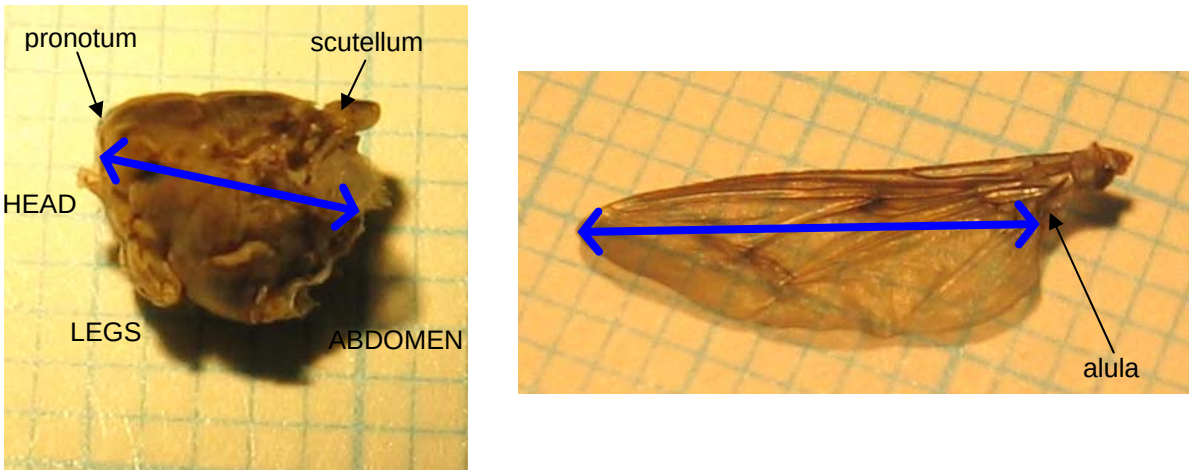

Figure 1. Photos of cut thorax (A) and cut wing (B) of *G. brevipalpis*

Measurements were taken as shown by the blue arrows, from beneath the fold of the scutellum to the front face of the pronotum for the thorax, and from the distal base of the fold of the alula to the tip of the wing. Each square of the background measures 1mm square.

Female tsetse flies are slightly bigger than males as shown in the figure 2, and it is the abdomen of the female which is longer, as well as the wings.

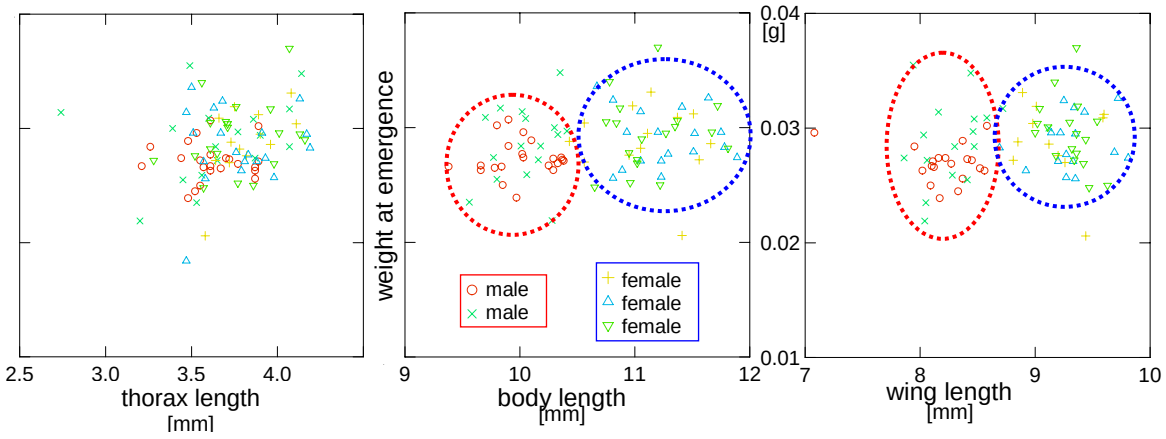

Figure 2. Comparison between the thorax, body and wing lengths as a function of the weight at emergence for different groups of *G. pallidipes* from rearings (n=48 males and 68 females).

The legend on the ordinate (*weight at emergence*) is common to the three panels of the figure.

As newly emerged teneral tsetse flies have their flight muscles incompletely developed, their flight duration is restricted. During maturation these thoracic muscles increase in size (Langley, 1970; Langley *et al.*, 1990) as well as the proline reserves used as an energy source during flight. This muscle increase can be observed by weighting the insect before each meal (Fig. 3).

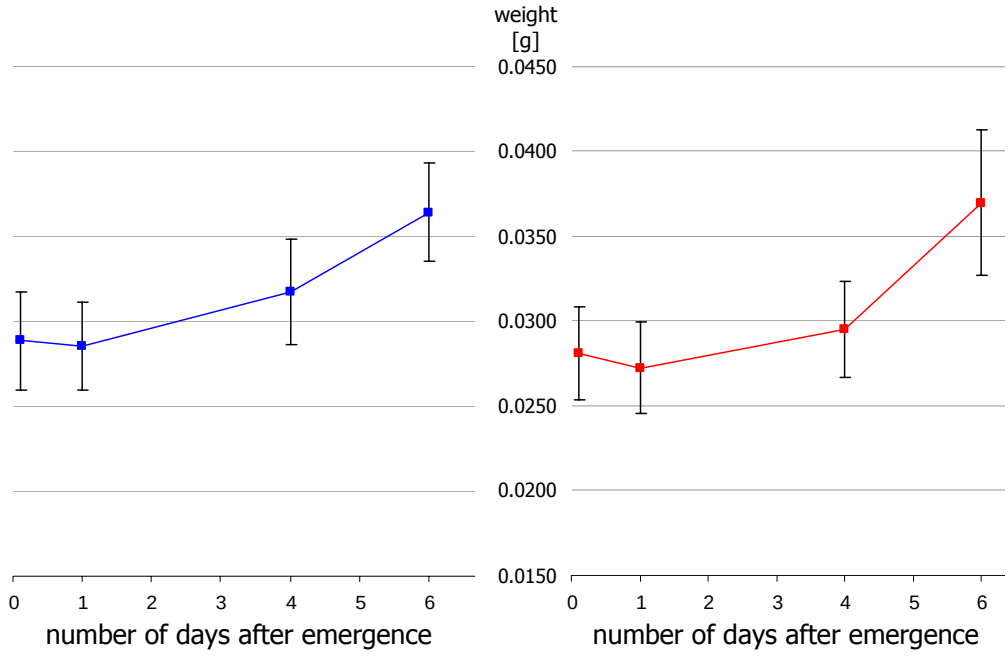

Figure 3. Progression in the weight gains of female and male *G. pallidipes* fed at regular intervals.

Before each meal on days 1, 4 and 6 after emergence, females (blue curve ; n=68, 68, 61, 19 flies) and males (red curve ; n=48, 48, 41, 17) were weighed.

Both sexes of tsetse flies are strictly haematophagous and their blood meal provides all the energy, protein and water needed for homeostasis and reproduction (Gibson and Torr 1999). With some exceptions, the best inter-meal period is 2-3 days for laboratory reared tsetse flies, even though the interval required between meals depends on the tsetse fly species and rearing temperature (Bursell & P., 1980; Langley & Stafford, 1990; Gaston & Randolph, 1993). Moreover, it was shown that the quantity of blood intake increases at each meal and can reach the weight of the insect (Leak 1999). Similar results were obtained for *G. pallidipes* in our insectarium (Fig. 4).

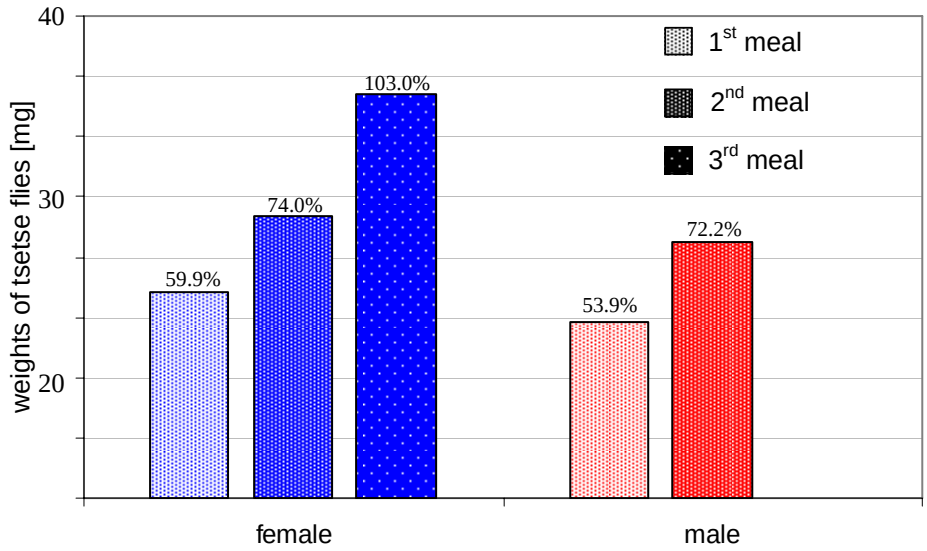

Figure 4. Progression in the amounts of blood taken at each meal for female and male *G. pallidipes*. Flies were weighed before and after each meal taken on days 1, 4 and 6 after emergence. The percentage corresponds to the amount of blood imbibed relative to the weight of the insect before the blood meal (n=61, 57 and 19 females and n=42 and 42 males).

The hunger state of a tsetse fly is correlated to the fat body reserves (Langley, 1970; Langley & Wall, 1990). Figure 5 shows that the decrease in lipid reserves in the abdomens of *G. pallidipes* is linked to the number of days of starvation in our insectarium.

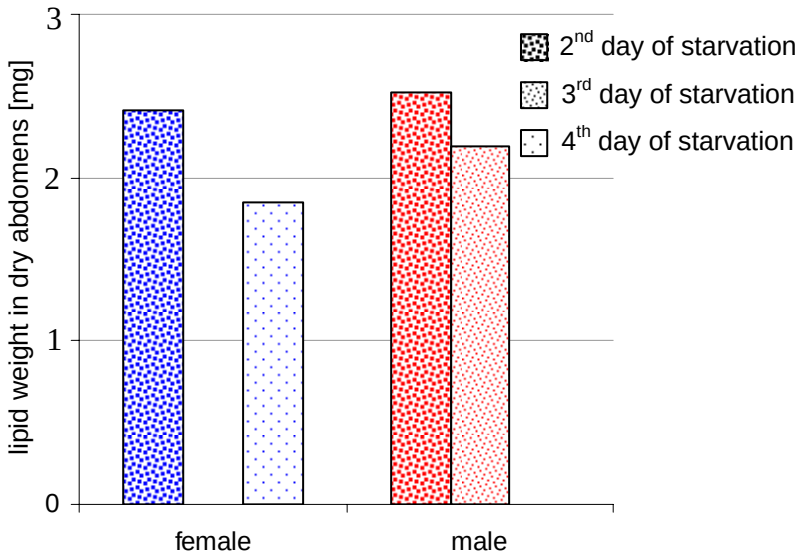

Figure 5. Decrease in the amount of lipid reserves in *G. pallidipes* abdomens depending on the number of days of starvation (n=19 and 18 females, and 17 and 23 males).

Furthermore, the amount of lipid reserves in *G. pallidipes* abdomens can be correlated with the appetence behaviours of the flies in the wind-tunnel when stimulated by breath (Fig 6). Breath is a strong attractant for tsetse flies (*Chapter 3*) and the propensity of tsetse flies to undertake the different steps of activation, exit the release cage, directed flight and attraction to the source is influenced by the amount of lipid reserves in the abdomen: the higher the reserves the lower the propensity to fly to the source.

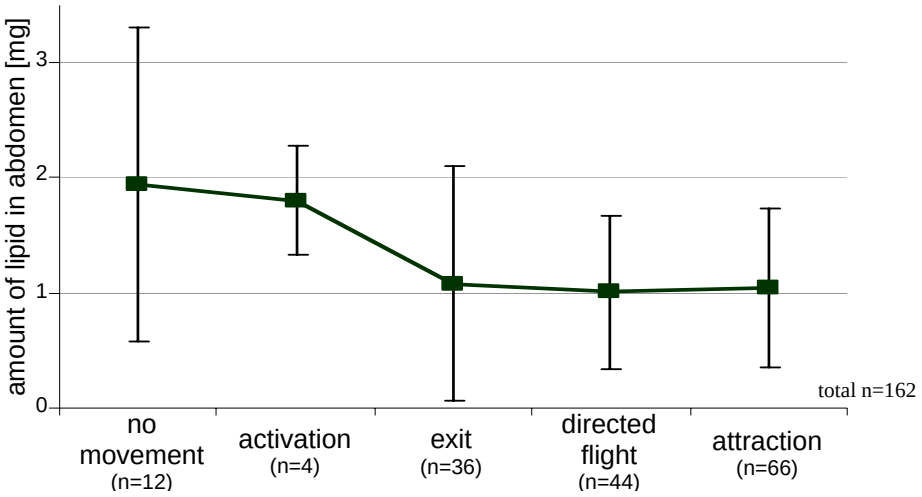

Figure 6. Relation between quantity of lipid reserves in *G. pallidipes* abdomens (n=162) and appetence behaviours when stimulated by human breath in the wind tunnel.

Despite the fact that very few insects did not respond to breath, those that did not do so were also those with the highest amount of abdominal lipids. Under each behaviour criterion is the number of insects that terminated the experiment under the criterion. The 12 insects that showed no movement and the 4 that only showed activation within the release cage were also those with the highest lipid reserves. All the flies that undertook release cage exit, directed flights and attraction to human breath had lower lipid reserves.

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ANNEX D - Tsetse flies produce sounds

Sound production and reception are far less studied than is the role of chemical signals in communication (Erickson and Møller 1975). It has been known for quite some time that various species of tsetse fly produce sounds while in flight (Kartman, Campau et al. 1946) or when resting before and after a meal (Hegh, 1929 in Kartman, Campau et al. 1946). Sound has consequently been suggested as a mean of communication (Kartman *et al.*, 1946; Vanderplank, 1948; Glover, 1967), which might be responsible for the aggregations in following swarms described by Swynnerton (Wynne-Edwards 1962), or in relation to "social behaviour" with a negative correlation between the number of flies in the cage and the probability of producing sound (Popham, Parr et al. 1978). As reviewed by Wall & Langley (1993) even though tsetse populations exist at very low densities behavioural mechanisms leading to meeting of the sexes and timely successful inseminations are highly efficient. Some authors consider that singing is a spontaneous activity and not a by-product of other activity, even if sound production can be controlled by exogenous factors (Kartman *et al.*, 1946; Kolbe, 1974; Saini, 1981b; Saini, 1981a).

The low frequency sounds of tsetse flies are mainly produced by vibrations of the pterothorax area (Kolbe, 1974; Popham *et al.*, 1978; Chowdhury & Parr, 1981; Denlinger *et al.*, 1983) and some of the ultrasonic components may be produced by air pulsed out of the spiracles (Anderson 1978). Popham *et al.* (1978) concluded after experiments of selective antennectomy that the organ for perceiving sounds in tsetse is situated in the flagellum.

The post-feeding sounds produced for up to 30 minutes after feeding in tsetse flies are bursts lasting 2-30 seconds in duration showing slight variations in pitch and tone (Popham, Parr et al. 1978). The 2 commonest post-feeding sound patterns are, as defined by Kolbe (1974), the "calls" which are a short series of sounds of variable durations and intervals (Fig. 1) and the "songs" which are long continuous patterns of unmodulated sound, as recorded in this study with Cool Edit Pro Software (Syntrillium Software, USA) for *G. pallidipes* (Fig. 2).

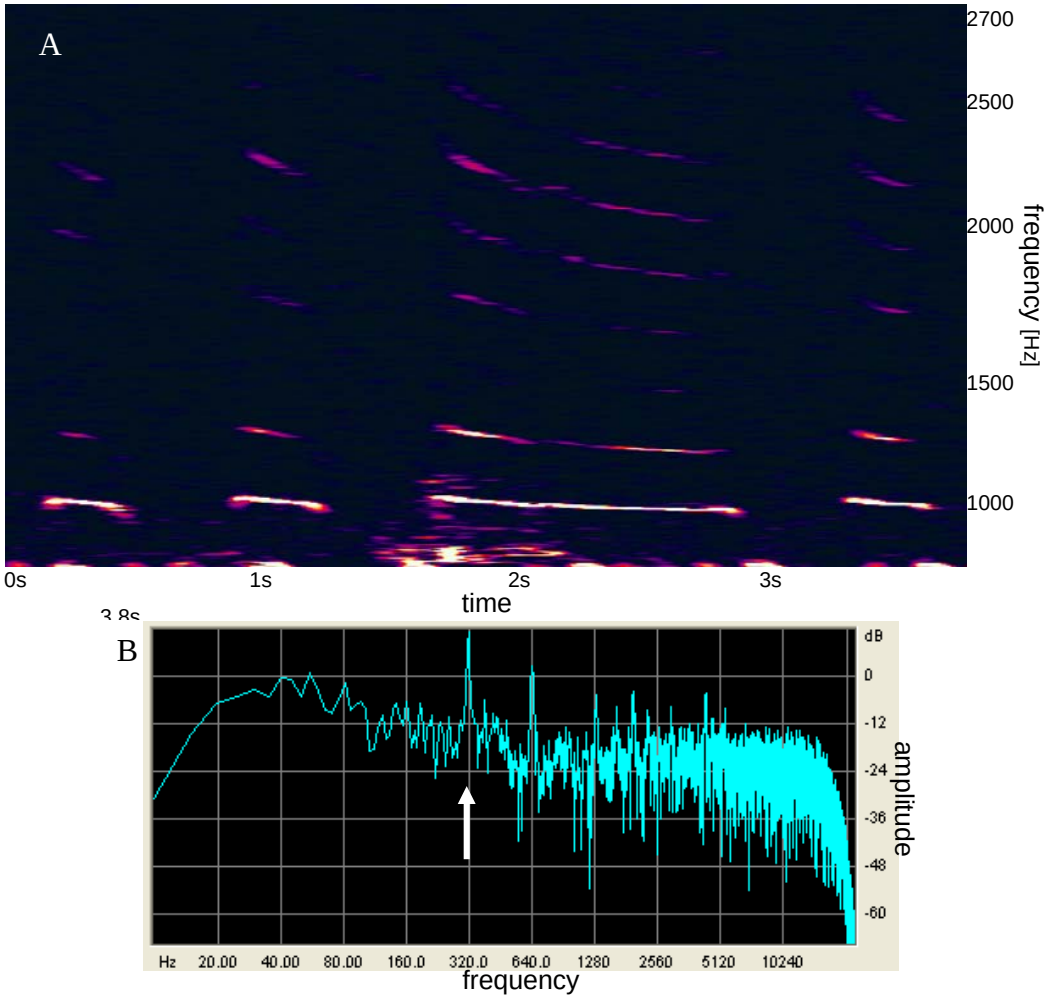

Figure 1. Spectrogram (A) and frequency spectrum (B) of the "call" of a fully fed *G. pallidipes* male recorded during this study.

The spectrogram represents the development of the sound frequency (vertical axis) as a function of time (horizontal axis). The intensity of the fundamental amplitude (bottom trace) and 4 to 6 harmonics (upper traces) are indicated by the colour variations from black through the different colour (violet, red, yellow) to white. The spectrogram shows a continuous recording with 3 short "calls" (≈0.4s) and 1 longer one (≈1.3s) with a fundamental frequency and 4 to 6 distinct harmonics. Spectra below 300Hz are noise.
The frequency spectrum (below) represents the sound amplitude in dB (vertical axis) of the frequency (horizontal axis) at a given time. At this time point the call analysed contained 5 major frequency peaks with the fundamental tone at ≈320Hz (arrow).

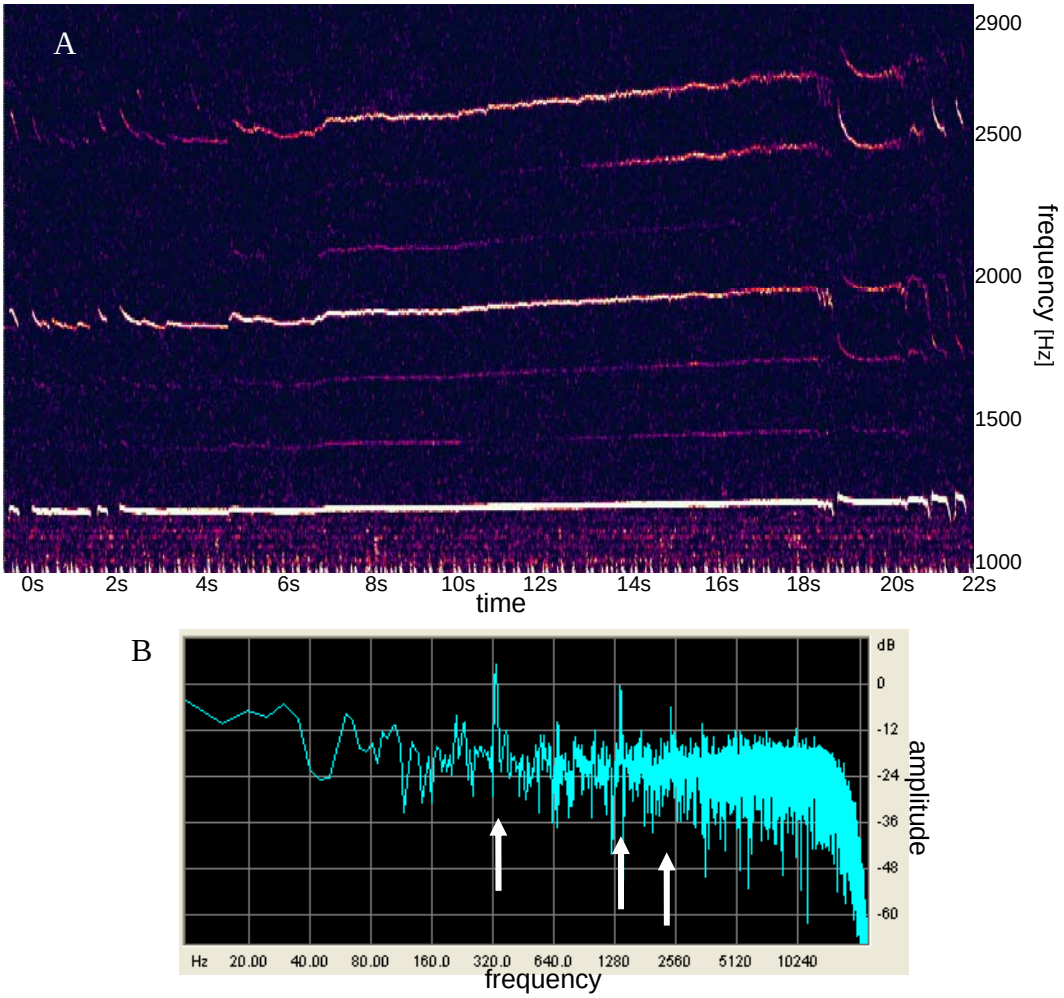

Figure 2. Spectrogram (A) and frequency spectrum (B) of the "song" of a fully fed *G. pallidipes* male

The spectrogram shows the recording of a song for ≈16s with 7 different frequencies represented but with 3 dominating frequencies

The frequency spectrum (below) shows the 3 dominant frequencies at ≈320Hz, ≈1300Hz and ≈2500Hz (arrows). Contrary to the "call" signal (*Figure 1*), the intensity in dB of the 1st overtone at 650Hz is low. For further details see legend to *Figure 1*.

The different sounds varied considerably in pitch, tone, pattern and duration between individuals (Kolbe 1974), and can be produced by flies either in solo (Fig. 1 & 2) or in concert (Fig. 3 & 4).

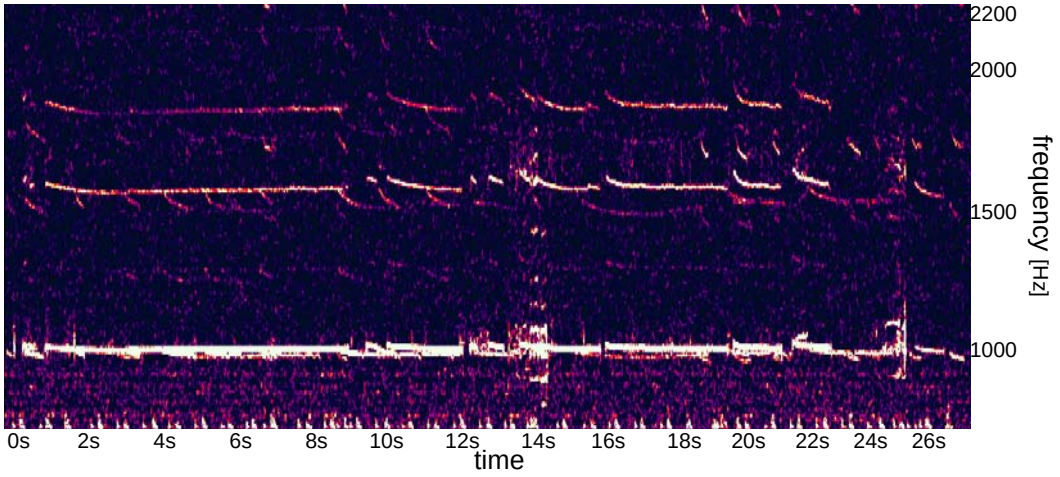

Figure 3. Spectrogram of "songs" and "calls" of *G. pallidipes* males produced in concert

The spectrogram represents the simultaneous recording of "songs" and "calls" against the background (less clear). Note the presence of the 1st overtone (just behind 1000Hz) only for the "calls" and the large fundamental tone due to superimposition of the 2 songs.
For further details see legend to *Figure 1*.

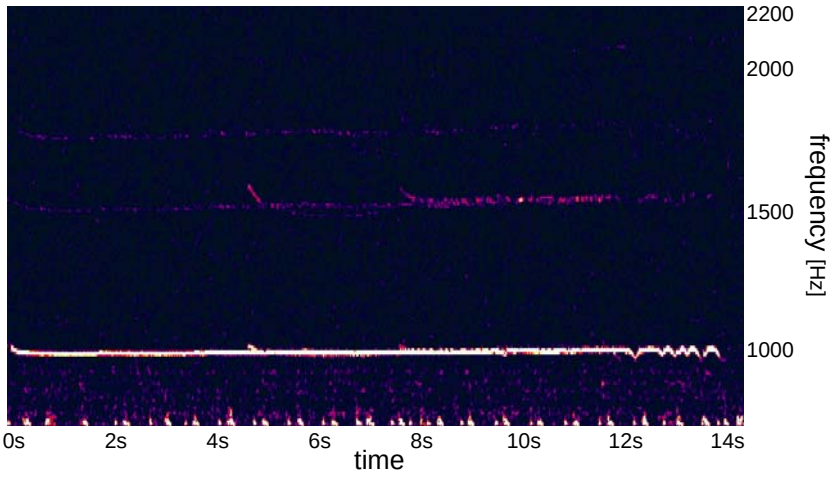

Figure 4. Spectrogram of 2 simultaneous "songs" of *G. pallidipes* males produced in concert

The spectrogram represents the recording of one male "song" joined at 4.5s and at 8s by a second male. Note how the second male seemed to tune up his "song" to the same frequency as the 1st one. For further details see legend to *Figure 1*.

Erickson & Møller (1975) detected harmonics up to 100kHz in tsetse, which may carry important sexual information as these high frequencies are different between females and males. During the precopulatory period, sound emission increased (Rudrauf 1977) and Vanderplank (1948) had observed that the mating calls stimulate copulation. Moreover, these calls may permit species and sex recognition by the female as suggested by Wall & Langley (1993). Moreover, Delinger *et al.* (1983) reported a unique pattern of sound production that immediately follows parturition. This post-partum song may permit to reorder the abdominal organs following displacement by the larva. Delinger *et al.* (1983) hypothesised too, that the song can have a signalling function for other pregnant females which could explain the pupae aggregations frequently encountered in the field.

Because distension of the abdomen seems to render the flies more prone to sing (Kolbe 1974), Howe & Lehane (1986) hypothesised that thorax vibrations permit the flies to accelerate and improve digestion of the blood meal. But laboratory studies have shown that tsetse react to sounds produced by other flies: males moved or called when teneral females sang (Kolbe 1974), and unfed flies perceived and reacted to the sound produced by gorged flies of the same species placed in an another cage (Popham, Parr et al. 1978). If sounds eventually prove to be an aggregation stimulus, then it may be possible to improve the performance of trapping devices by incorporating sound recordings. The advantages of this method would be that resting flies would be stimulated whereas visual stimuli alone do not attract them, and the range over which traps are effective might be larger since sound is an undulatory phenomenon transmitted in an isodiametric way, while odour-baited traps attract tsetse from the upwind direction only (Challier 1982).

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Annex E - Solute enrichment through large volume injection in gas chromatography

A good solution to better detect compounds present in small amounts in a sample by capillary gas chromatography is to inject a larger volume of solution. However this can cause a large solvent peak which can cover a big part of the chromatogram. To avoid this two methods of large volume injection (LVI) were developed allowing injections of up to 250µL of solution.

The first method consists of the addition of suitable retention gap made of few meters of deactivated non-coated fused silica capillary column at the head of the analytical column.

We added a 10m retention gap (0.32mm i.d.) just after the on-column injector and before the chromatographic column (30m, 0.250mm i.d., FFAP, BGB, Switzerland) and adjusted the carrier gas through the column to its optimum. To test the system, the polar Grob test mixture (Fluka, puriss p.a.) was injected at 3 different amounts (1µL, 10µL and 30µL) and responses of the flame ionisation detector (FID) to solutes was recorded on an integrator (SP4270, Spectra Physics, USA) as peak area (Tab. 1). While the proportions of amounts of solutes recovered between the 1µL injection, the 10µL and the 30µL injections are more or less equivalent, the amounts climbed proportional to the quantity injected. When 30µL of solution were injected the undecane peak was already masked by the solvent peak.

Table 1. Comparison of the amounts of compounds in the Grob polar test mixture recovered using a long retention gap for large volume injection.

	1µL		10µL		30µL	
	area	ratio	area	ratio	area	ratio
decane	0		0		0	
undecane	16936	1	238961	14	0	0
nonanal	14771	1	189634	13	439247	30
2,3-butanediol	9578	1	137150	14	263526	28
1-octanol	12296	1	164235	13	333992	27
C10 methyl ester	10460	1	146043	14	355790	34
C11 methyl ester	10645	1	140997	13	362327	34
C12 methyl ester	9628	1	135716	14	328801	34
2,6-dimethyl aniline	11633	1	156233	13	336373	29
2,6-dimethyl phenol	6618	1	116080	18	278392	42
2 ethyl hexanoic acid	5534	1	100054	18	261610	47

The quantity (*integrated area of peak*) of products detected by the flame ionisation detector was calculated using an integrator (SP4270, Spectra Physics, USA). The ratio of each compound was calculated by dividing the area measured for each solute with the 10µL and 30µL injection volumes by the areas measured for the 1µL injection.

The second solute enrichment technique uses the special split-splitless injector made by VOGT *et al.* (1979). Slight changes were made on their protocol because of our Gas-chromatograph (5300, Carlo Erba Instruments, Italy) equipped with a cold injection system (CIS 3, Gerstel®, Germany). Here the sample is slowly injected into the injector set at a temperature just below the solvent boiling point. During the injection, the splitter valve is fully open (split ratio of 1:600) and the solvent is flushed by the carrier gas through the vent-off valve. As advised by Grob (1988), if the speed of injection corresponds to the speed of solvent evaporation, only the sample constituents are trapped and concentrate in the glass insert filled with glass wool placed inside the injector. After a few seconds, the splitter valve is closed and the injector is quickly heated (12°C/s in our case) permitting a rapid transfer of the compounds to the top of the chromatographic column.

The CIS injection system of Gerstel® permits injections of larger volume with the programme "Solvent Venting – Stop Flow". We tried to find the best condition of injection, and chose, where as advised by Gerstel®, a low injection temperature (injector put at 0°C) with a high flow 100mL/min. The most important parameter of this programme seems to be the purge time (permitting the evaporation of solvent and volatile compounds), and the split time (during which compounds and solvent enter the chromatography column).

Using the parameters mentioned above, we injected 2 samples of the Grob polar test mixture: 10µL of a 1/10 dilution and 100µL of a 1/100 dilution in dichloromethane. The solvent peaks were not too big and the other compounds were recovered at relatively the same quantities between the 2 injections (Tab. 2). However, the lighter solutes disappeared and we assume that during injection they evaporated and were flushed out by the carrier gas with the solvent. The heavier compounds were recovered at lower amounts and we assume that the glass liner placed inside the injector retained them, a factor that led to a thermal degradation during the rapid injector heating. Moreover, as advised by Gerstel®, due to a large loss during the split period and thermal degradation during injector heating, the amount needed for detection by the FID increases from 0.5ppm to 50ppm.

Table 2. Comparison of the amounts of solutes in the Grob polar test mixture recovered by the split-splitless large volume injection.

	10µL of 1/10 dilution		100µL of 1/100 dilution	
	area	ratio	area	ratio
decane	0		0	
undecane	0		0	
nonanal	0		0	
2,3-butanediol	4626	1	0	0
1-octanol	10891	1	10262	0.94
C10 methyl ester	9071	1	8144	0.90
C11 methyl ester	13995	1	8078	0.58
C12 methyl ester	10563	1	8168	0.77
2,6-dimethyl aniline	7583	1	11985	1.58
2,6-dimethyl phenol	6101	1	9177	1.50
2 ethyl hexanoic acid	2227	1	3015	1.35

The quantity (*integrated area of peak*) of products detected by the flame ionisation detector was calculated using an integrator (SP4270, Spectra Physics, USA). The ratio of each compound detected was calculated by dividing the area measured with the 100µL injection by the area measured with 10µL injection.

To conclude, comparison between the two techniques of LVI tested showed that none of them permit us to recover the low boiling solutes injected as they are either masked by the large solvent peak when using the long retention gap or are flushed with the solvent with the large volume split-splitless injector system. The amount of solutes recovered by both techniques was more or less equivalent, but the long retention gap method gave more consistent results (Fig. 1).

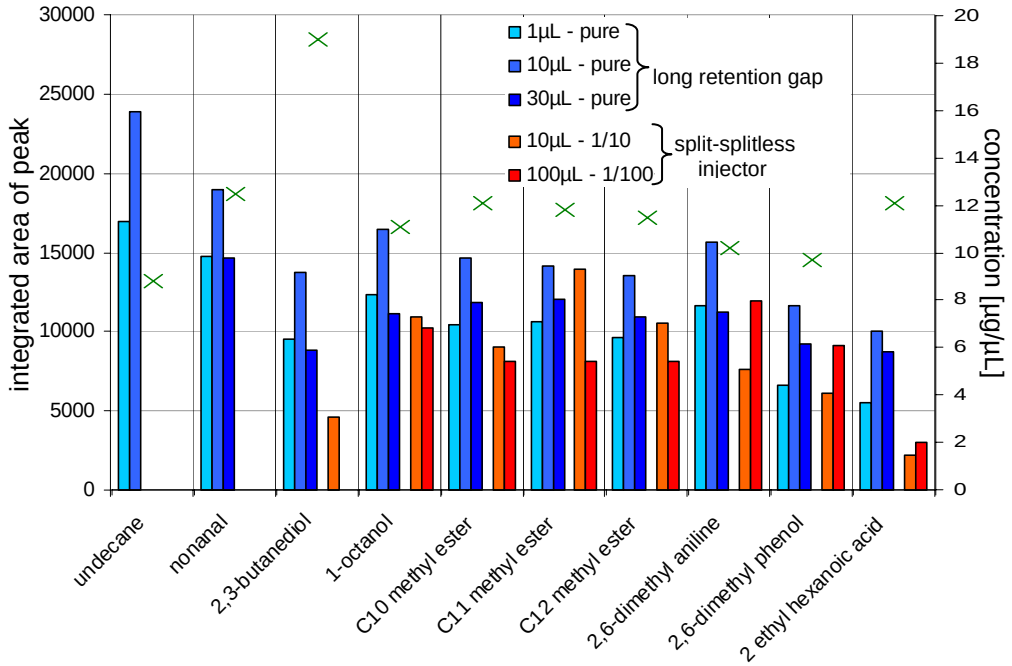


Figure 1. Amount of solutes recovered using the two large volume injection techniques and compared to theoretical concentrations.

The blue histograms (for the long retention gap method) and the red histograms (for the split-splitless injection method) represent the amounts of solutes recovered. All the quantities by peak integration were calculated using an integrator (SP4270, Spectra Physics, USA), and then normalised to obtain a ratio relative to injection of 1µL of the neat Grob test mixture. The green crosses represent the theoretical concentration of solutes present in the polar Grob mixture.

Large amount of the rumen fluid fraction containing neutral compounds and amines were analysed using the split-splitless injector method. However, even with injections of 5 and 10µL volumes the increase in the amounts of solutes was accompanied by an overall increase in the noise level (Fig. 2).

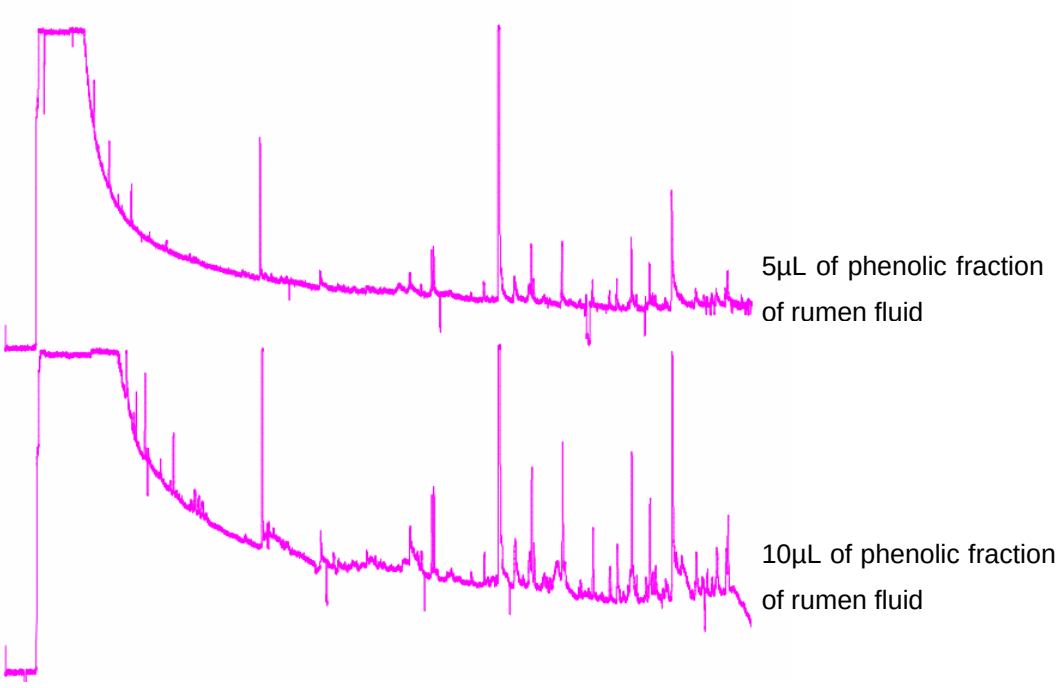
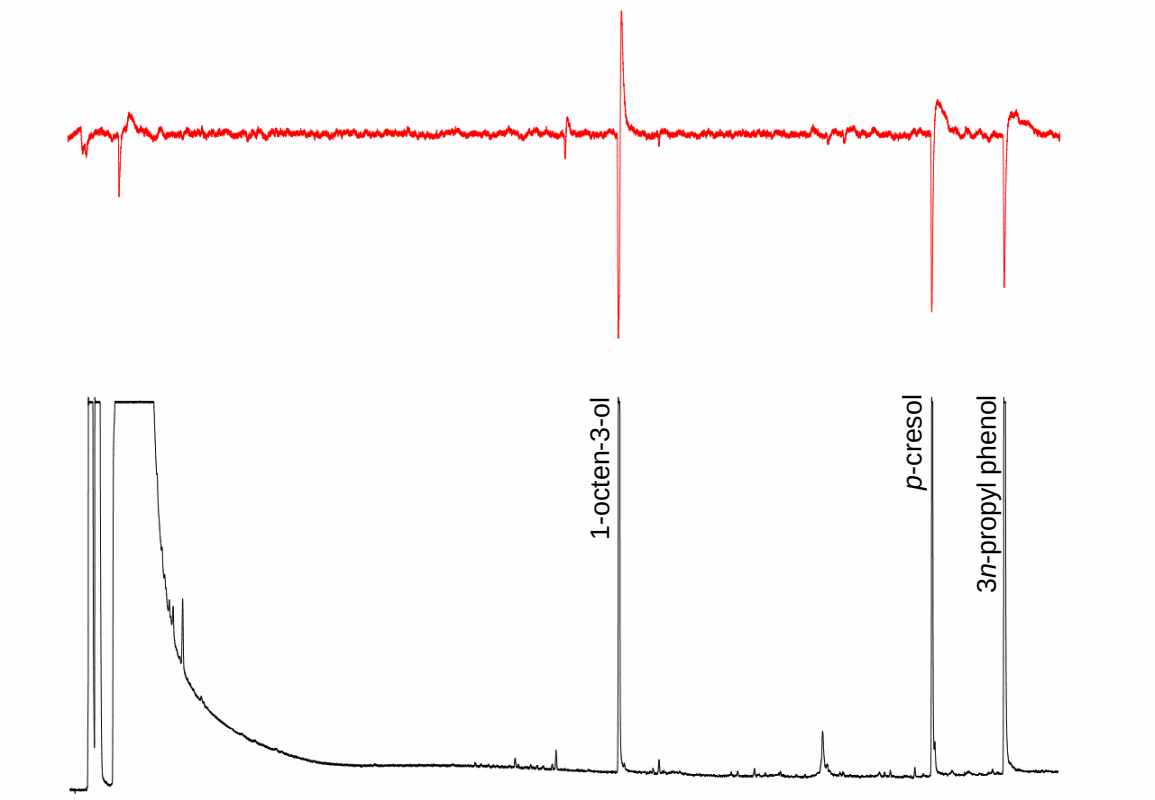


Figure 2. Flame ionisation detector responses generated during elution of the constituents present in 5µL and 10µL aliquots of the phenolic fraction of rumen fluid from the gas chromatographic column equipped with the Gerstel® split-splitless injector permitting LVI.

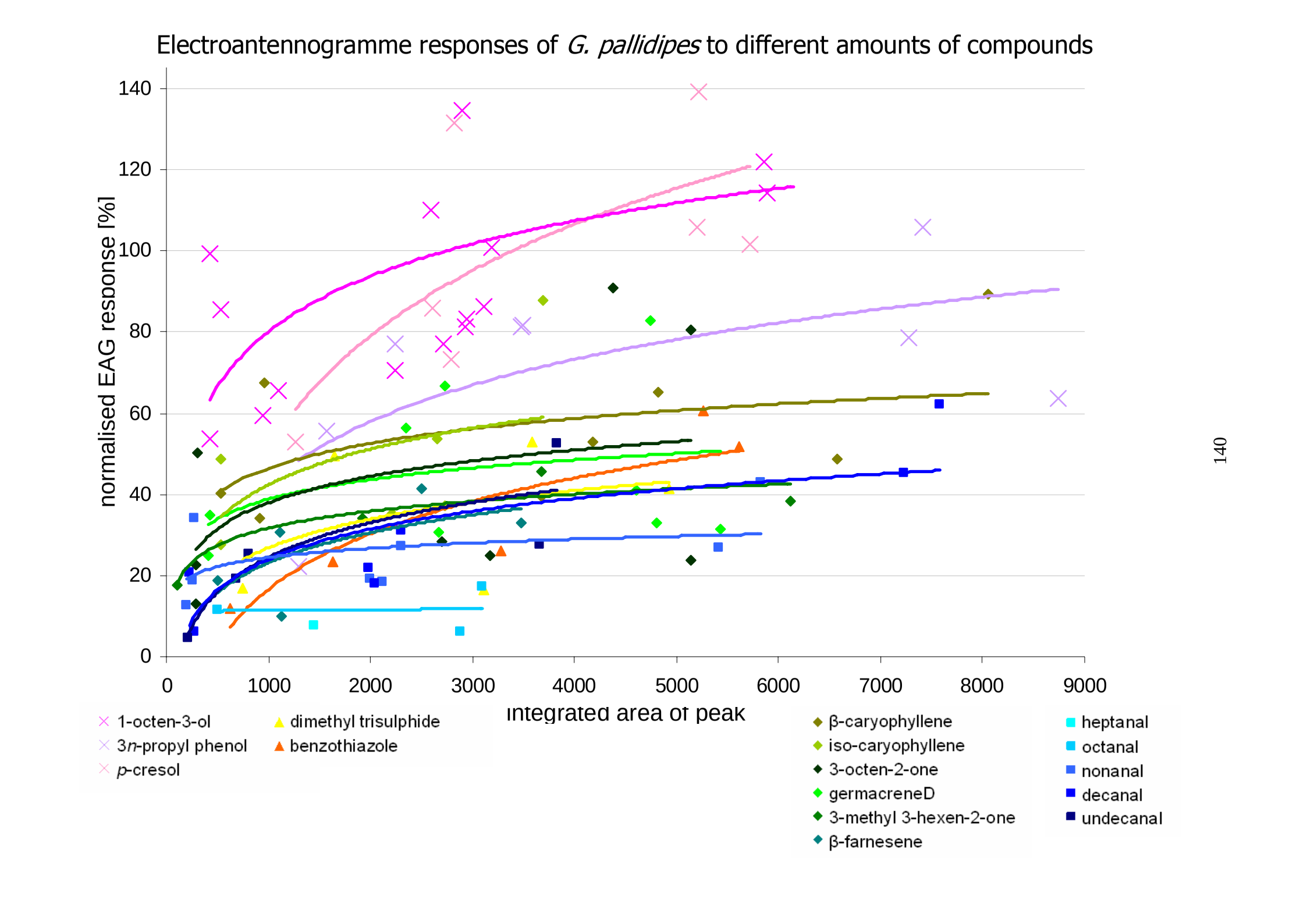
The injection method was by slow injection (at approximately 0.1µL/s) followed by a short purge time (split still open) of ~10s and a long splitless time of 2min during which the injector started to warm from 0°C to 230°C at a rate of 12°C/s.

ANNEX F - Electroantennogram responses of *G. pallidipes* in the laboratory

§

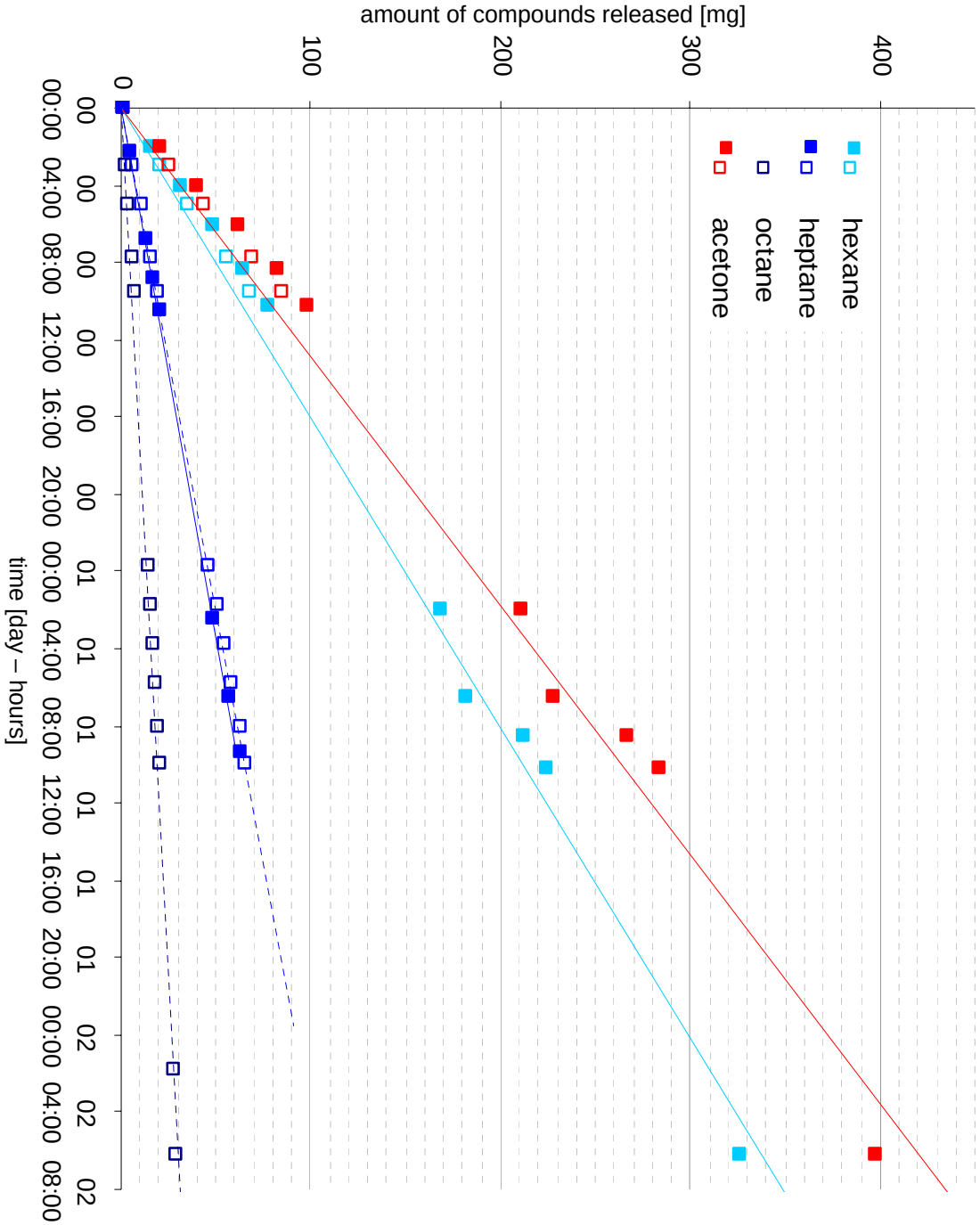


Electroantennogramme responses of *G. pallidipes* to 1-octen-3-ol, *p*-cresol and 3*n*-propyl phenol (used in the field as attractant) presented to the antenna by gas chromatography coupled EAG recordings.

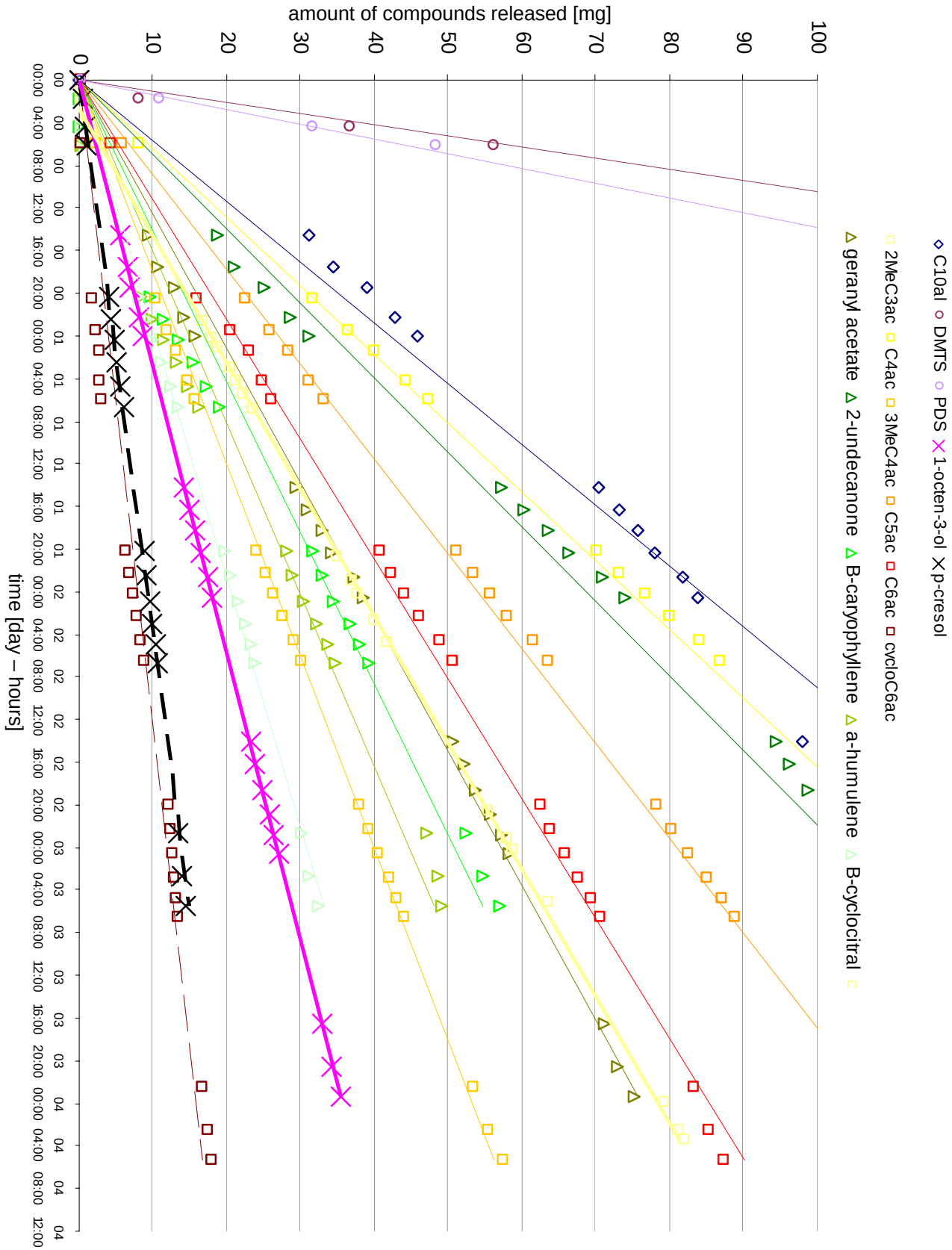

Electroantennogramme responses of G. pallidipes to different amounts of compounds
normalised EAG response [%]
0
20
40
60
80
100
120
140
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
integrated area of peak
1-octen-3-ol
3n-propyl phenol
p-cresol
dimethyl trisulphide
benzothiazole
β-caryophyllene
iso-caryophyllene
3-octen-2-one
germacreneD
3-methyl 3-hexen-2-one
β-farnesene
heptanal
octanal
nonanal
decanal
undecanal
140

ANNEX G - Measures of the release rate of different compounds

1mL (empty symbols) and 5mL (filled symbols) of hexane, heptane, octane and acetone were deposited neat inside a 1L gas-wash bottle. A mean evaporation rate was calculated by weightings over 2 consecutive days while placed in a fume hood with a draught (900m^3/h).



A polyethylene sachet (4cm×5.5cm, film thickness 150µm) was filled and sealed with 1mL of a compound. A mean evaporation rate was calculated by weightings over 4 consecutive days (n>20) while hanging in a fume hood with a draught (900m^3/h);



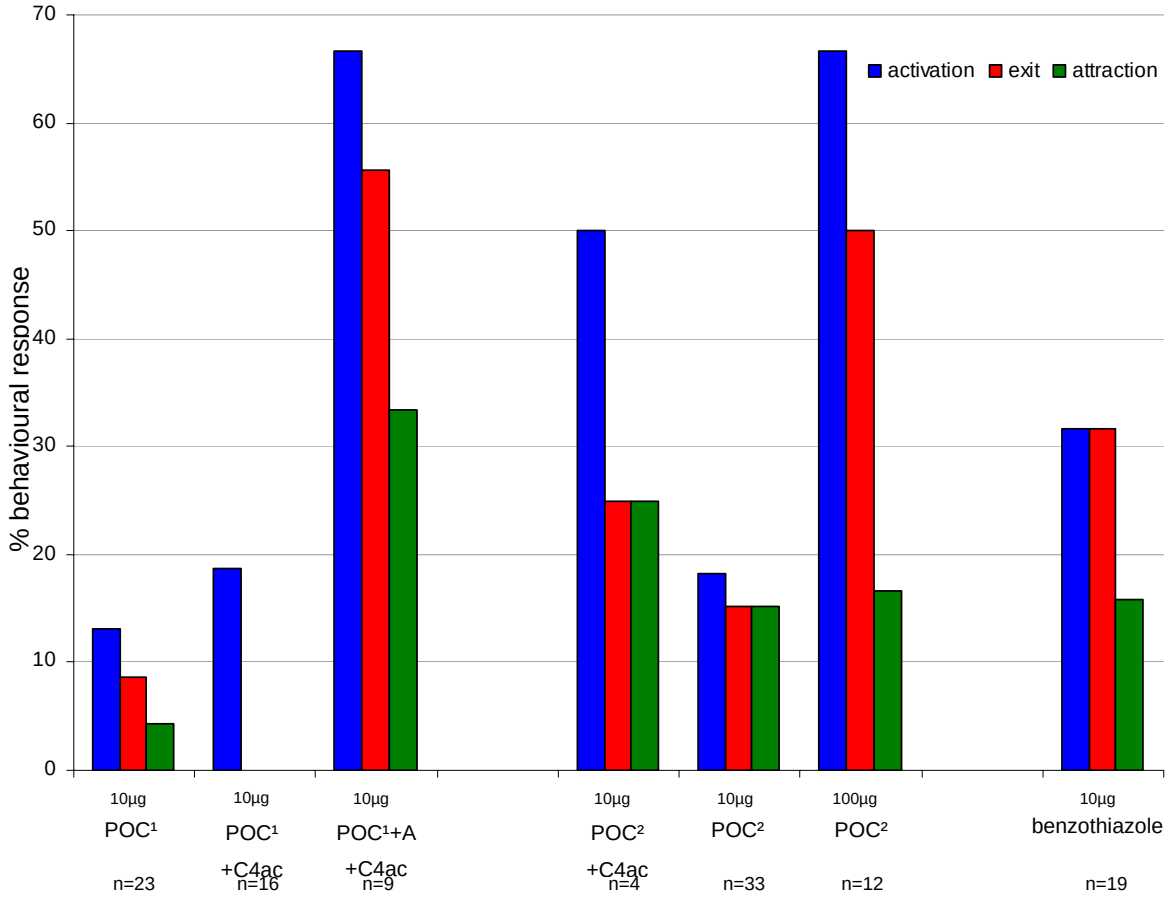
Annex H - Behavioural tests in the wind tunnel

Behavioural criteria recorded were: *activation*, the fly moved within the cage; *exit*, the fly flew out of the cage; *directed flight*, the fly travelled at least 50cm within the plume of odour; *attraction*, the fly made a directed flight to whithin 10cm of the source.

Behavioural responses of tsetse flies using 1L gas-wash bottle and filter paper disk

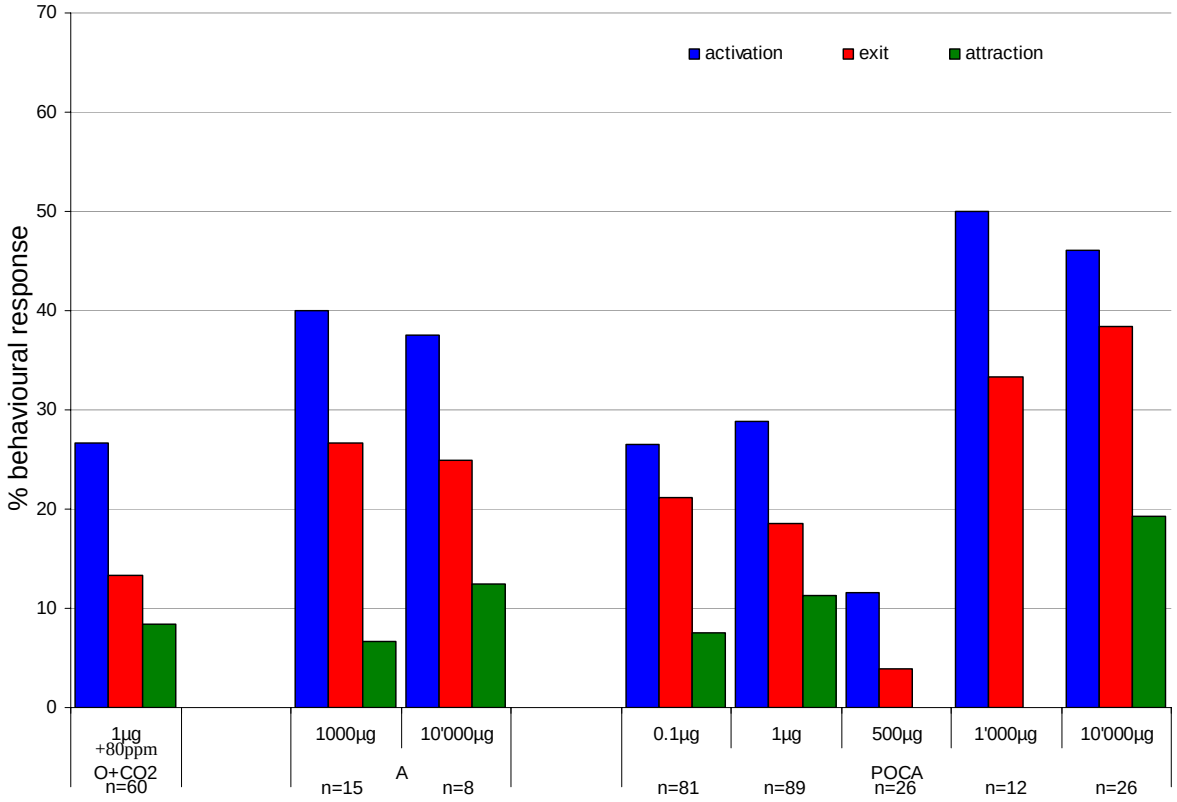
For all tests charcoal filtered air (0.5L/min) was used, passing through a 1L gas-wash bottle containing a filter paper disk (9cm diameter) to which test solution was applied (amount in µg written behind each histogram). Compounds were diluted in dichloromethane.
CO2: carbon dioxide ; P: 3n-propyl phenol ; O: 1-octen-3-ol ; C: p-cresol ; A: acetone ; C3ac: propanoic acid ; C4ac: butanoic acid ; 3MeC4ac: isovaleric acid ; C6ac: hexanoic acid.

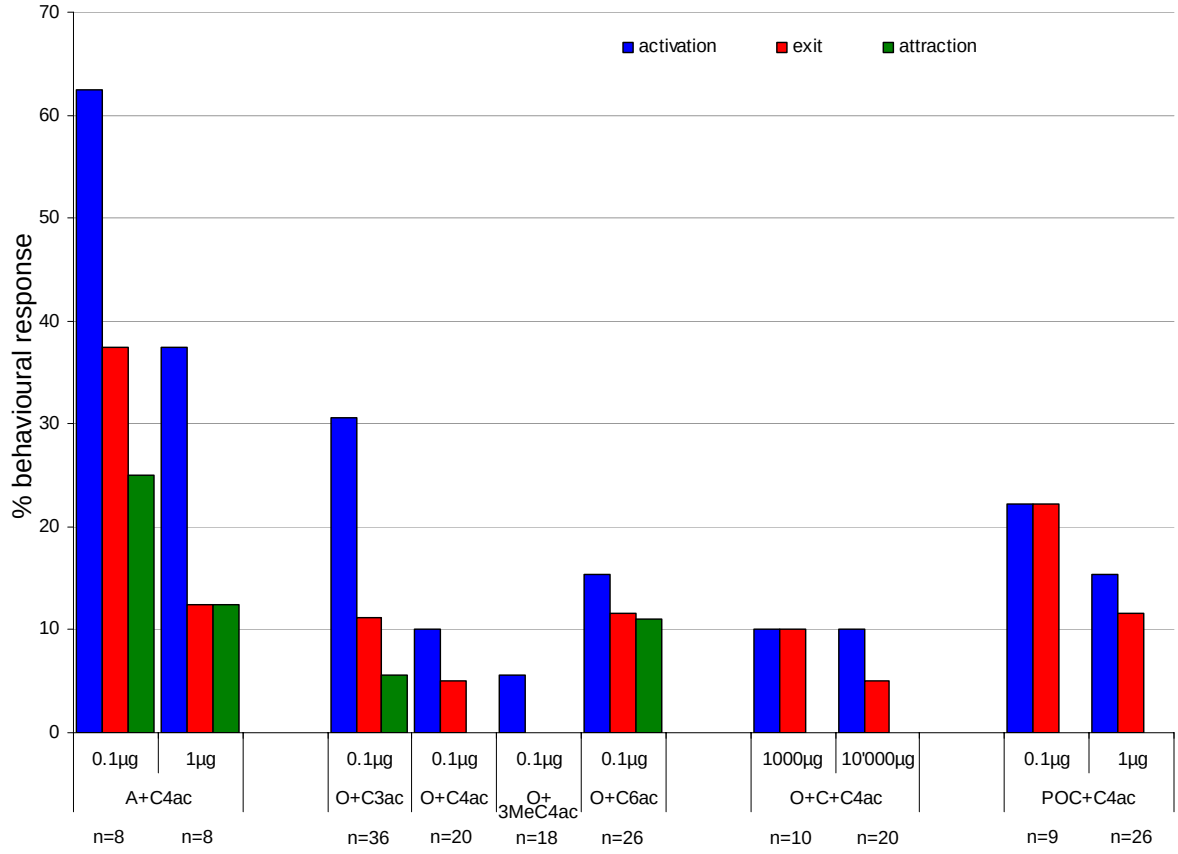
Behavioural responses of *G. pallidipes*



The mixture of 3*n*-propyl phenol, 1-octen-3-ol and *p*-cresol diluted in DCM was used at a ratio of 1:4:8 for POC^1 and 1:0.6:9 for POC^2 (by weight and head space collection gave a ratio of 1:8:24 and 1:28:8 respectively).

Behavioural responses of *G. brevipalpis*

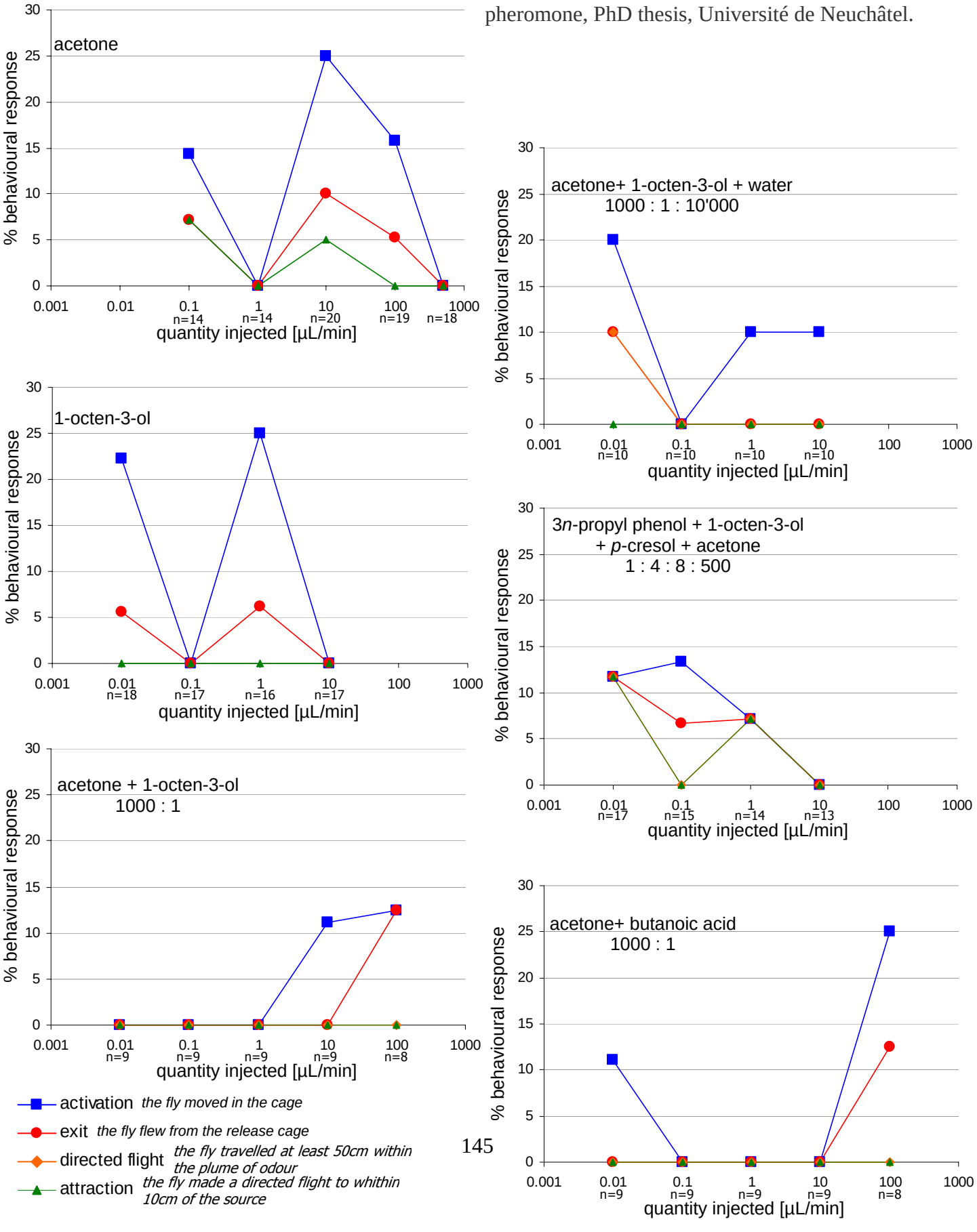




G. pallidipes behavioural responses using piezo sprayer as dispenser

For more details regarding the piezo nebulizer system, see EL SAYED, A., GODDE, J. & ARN, H. (1999). Sprayer for quantitative application of odor stimuli. *Environmental Entomology* **28,** 947-953.

For more details regarding the procedures, see SCHMIDT-BÜSSER, D. (2008). Host plant volatiles influence the behavioural responses of the European grape berry moth, *Eupoecilia ambiguella*, to its pheromone, PhD thesis, Université de Neuchâtel.



Documents

GENERAL INTRODUCTION

Photo of *G. brevipalpis* male fed

Source: Harraca V.

- **Table 1.** Host odour compounds and their combinations currently used to improve trap catches of tsetse fly spp. from different subgenera.
- **Figure 1.** Predicted areas of suitability for tsetse flies of the 3 subgenera.

Source: FAO

http://www.fao.org/Ag/AGAInfo/programmes/en/paat/documents/maps/pdf/Groups/AF_morsgr_V.pdf
http://www.fao.org/Ag/AGAInfo/programmes/en/paat/documents/maps/pdf/Groups/AF_palpgr_V.pdf
http://www.fao.org/Ag/AGAInfo/programmes/en/paat/documents/maps/pdf/Groups/AF_fuscgr_V.pdf.

- **Figure 2.** An example of chambered stomach of the cow.

Source: Harraca V.

- **Figure 3.** Map of the principal local foci of human sleeping sickness in Africa and dominant trypanosome species responsible for the disease.

Source**:** WHO

http://whqlibdoc.who.int/trs/WHO_TRS_881.pdf (Fig1)

- **Figure 4.** Number of reported cases of human African trypanosomiasis and population levels screened between 1940-1998.

Source**:** WHO

http://whqlibdoc.who.int/hq/2000/WHO_CDS_CSR_ISR_2000.1.pdf (Fig8.2)

- **Figure 5.** Photos with detail of a *G. brevipalpis* cut head during electroantennogram recording with glass capillary electrodes.

Source: Harraca V.

- **Figure 6.** Visualisation of a plume of odour in the wind tunnel used for these studies.

Source: Harraca V.

CHAPTER 1.

Photo of the wind tunnel with the blue visual targe tat upwind

Source: Gurba A.

- **Table 1.** Mean behavioural responses and corresponding standard deviation values over days for the responses of *G. brevipalpis* and *G. pallidipes* to different treatments.
- **Figure 1.** Upwind flight of *G. brevipalpis* interpolated at different step lengths.
- **Figure 2.** Progression of flight velocity without interpolation and with interpolation at different step lengths for a flight by *G. brevipalpis* along the lengths of the wind tunnel (from right to left).
- **Figure 3.** 3D representations of flights made by *G. brevipalpis* in response to human breath in the wind tunnel.
- **Figure 4.** Top view of upwind flights of *G. brevipalpis* responding to human breath in the wind tunnel.
- **Figure 5.** Side views of interpolated upwind flights made by *G. brevipalpis* stimulated with human breath in the wind tunnel.
- **Figure 6.** *G. brevipalpis* instantaneous ground speeds extrapolated at successive 5cm positions along the length of the wind tunnel (X) during first upwind and subsequent upwind flights when stimulated by human breath.
- **Figure 7.** Top and side views of first upwind and first downwind flights made by *G. brevipalpis* stimulated by human breath in the wind tunnel.
- **Figure 8.** Instantaneous straightness indices for first upwind (left) and first downwind flights (right) made by *G. brevipalpis* in response to breath in the wind tunnel extrapolated at successive 5cm positions along the length (X) of the wind tunnel.

CHAPTER 3.

Photo of EAG preparation with *G. pallidipes* head between the two glass electrodes
Source: Harraca V.

- **Table 1.** List of the most common or interesting volatile organic compounds found by diverse authors in human and/or cow breath.
- **Table 2.** List of the synthetic compounds used in GC-EAD injections and behavioural experiments.
- **Table 3.** Behavioural responses of G. brevipalpis in 6 successive experiments to human breath from a 1L gas-wash bottle and a 25L Tedlar® bag filled with human breath, and to CO_2 at 3 different concentrations.
- **Figure 1.** Behavioural responses of G. brevipalpis to 6 successive experiments with breath from a 1L gas-wash bottle and a Tedlar® bag filled with human breath.
- **Figure 2.** CO_2 levels measured at 5cm from the stimulus outlet in the plume of breath from a gas-wash bottle and a Tedlar® bag filled with breath at the beginning of the experiment.
- **Figure 3.** Behavioural responses of G. brevipalpis to stimulation with comparable CO_2 concentration in breath as delivered from a 1L gas-wash bottle and a Tedlar® bag, and from a gas tank.
- **Figure 4.** Electroantennogramme responses of G. pallidipes to 100ng doses of synthetic aldehydes injected into a gas chromatographic column.
- **Figure 5.** Electroantennogramme responses of G. pallidipes to different amounts of synthetic aldehydes presented to the antenna by gas chromatography coupled EAG recording.
- **Figure 6.** EAG of G. pallidipes to a mixture of terpenes presented at different amounts to the antenna by gas chromatography coupled EAG recording.
- **Figure 7.** Electroantennogramme responses of G. pallidipes to different amounts of synthetic terpenes and a ketone presented to the antenna by gas chromatography coupled EAG recordings.
- **Figure 8.** EAG responses of G. brevipalpis, G. fuscipes & G. pallidipes, to synthetic ketones and a terpene presented at different concentrations in air passing over the antenna.
- **Figure 9.** EAG responses of G. pallidipes and G. fuscipes to different amounts of dimethyl trisulfide eluting from a gas chromatographic column.
- **Figure 10.** EAG responses of G. pallidipes and G. fuscipes to different amounts of dimethyl disulfide, dimethyl trisulfide and 1-octen-3-ol eluting from a gas chromatographic column.
- **Figure 11.** EAG responses of G. pallidipes and G. brevipalpis to benzothiazole and dimethyl trisulfide presented at different amounts to the antennae by gas chromatography coupled EAG recording.

CHAPTER 4.

Photo of 3D tracking system cameras
Source: Harraca V.

- **Table 1.** Release rate of the compounds used in the behavioural experiments.
- **Table 2.** Summary of the behavioural responses recorded in the wind tunnel for G. brevipalpis in response to acetone, hexane, heptane or octane alone and to these 4 products tested with 1-octen-3-ol or the ternary mixture of 3n-propylphenol, p-cresol and 1-octen-3-ol used in a polyethylene sachet.
- **Table 3.** Partial list of alkanes found by diverse authors in breath of human and cattle.
- **Figure 1.** Pathway of pentane formation from linoleic acid hydroperoxyde decomposition.
- **Figure 2.** Mean electroantennogramme responses of G brevipalpis to acetone, C_5 to C_{10} alkanes and dichloromethane at different concentrations in the air.
- **Figure 3.** Mean electroantennogramme responses of G pallidipes to acetone, C_5 to C_{10} alkanes and dichloromethane at different concentrations in the air.

- **Figure 4.** Behavioural responses of G. brevipalpis tested for responses to acetone and alkanes in the wind tunnel.
- **Figure 5.** Behavioural responses of G. brevipalpis to acetone, hexane, heptane or octane plus 1-octen-3-ol and delivered into the odour plume in the wind tunnel.
- **Figure 6.** Behavioural responses of G. brevipalpis in the wind tunnel to acetone, hexane, heptane and octane added to the tsetse fly attractants 3n-propylphenol, 1-octen-3-ol and p-cresol as released from a polyethylene sachet.
- **Figure 7.** Behavioural responses of G. brevipalpis in the wind tunnel by heptane, heptane plus 1-octen-3-ol and heptane plus 1-octen-3-ol, 3n-propylphenol and p-cresol.

GENERAL DISCUSSION

Photo of *G. brevipalpis* fed

Source: Harraca V.

- **Figure 1.** Different pathways of linoleic acid degradation.
- **Figure 2.** Possible interchanges between compound families through oxidation/reduction reactions.
- **Figure 3.** Structural similarities between compounds formed through amino acid degradation.

ANNEXES

Photos of *G. brevipalpis* during blood meal feeding through a silicon membrane

Source: Harraca V.

ANNEX A

- **Figure 1.** Rate of emergence of *G. brevipalpis* and *G. pallidipes* as a function of temperature.
- **Figure 2.** Rate of emergence of *G. brevipalpis* female and male linked to time.
- **Figure 3.** Relation between time spent at 12°C and the rate of emergence of 3 different tsetse fly species.

ANNEX B

- **Figure 1.** Frequency of movements by *G. pallidipes* over the day.
- **Figure 2.** Measurements of *G. pallidipes* activity over several days.

ANNEX C

- **Figure 1.** Photos of cut thorax (A) and cut wing (B) of *G. brevipalpis*

Source: Harraca V.

- **Figure 2.** Comparison between the thorax, body and wing lengths as a function of the weight at emergence for different groups of *G. pallidipes* from rearings (n=48 males and 68 females).
- **Figure 3.** Progression in the weight gains of female and male *G. pallidipes* fed at regular intervals.
- **Figure 4.** Progression in the amounts of blood taken at each meal for female and male *G. pallidipes.*
- **Figure 5.** Decrease in the amount of lipid reserves in *G. pallidipes* abdomens depending on the number of days of starvation (n=19 and 18 females, and 17 and 23 males).
- **Figure 6.** Relation between quantity of lipid reserves in *G. pallidipes* abdomens (n=162) and appetence behaviours when stimilutated by human breath in the wind tunnel.

ANNEX D

- **Figure 1.** Spectrogram (A) and frequency spectrum (B) of the "call" of a fully fed *G. pallidipes* male recorded during this study.
- **Figure 2.** Spectrogram (A) and frequency spectrum (B) of the "song" of a fully fed *G. pallidipes* male
- **Figure 3.** Spectrogram of "songs" and "calls" of *G. pallidipes* males produced in concert
- **Figure 4.** Spectrogram of 2 simultaneous "songs" of *G. pallidipes* males produced in concert

ANNEX E

- **Table 1.** Comparison of the amounts of compounds in the Grob polar test mixture recovered using a long retention gap for large volume injection.
- **Table 2.** Comparison of the amounts of solutes in the Grob polar test mixture recovered by the split-splitless large volume injection.
- **Figure 1.** Amount of solutes recovered using the two large volume injection techniques and compared to theoretical concentrations.
- **Figure 2.** Flame ionisation detector responses generated during elution of the constituents present in 5µL and 10µL aliquots of the phenolic fraction of rumen fluid from the gas chromatographic column equipped with the Gerstel® split-splitless injector permitting LVI.

ANNEX F

- Electroantennogramme responses of *G. pallidipes* to 1-octen-3-ol, *p*-cresol and 3*n*-propyl phenol (used in the field as attractant) presented to the antenna by gas chromatography coupled EAG recordings.
- Electroantennogramme responses of *G. pallidipes* to different amounts of compounds.

ANNEX G

- Release rate with gas wash bottle.
- Release rate with polyethylene sachet.

ANNEX H

- Wind tunnel behaviour of *G. pallidipes* using 1L gas wash bottle.
- Wind tunnel behaviour of *G. brevipalpis* using 1L gas wash bottle.
- Wind tunnel behaviour of *G. pallidipes* using piezo nebulizer.